Biological Control of Late Blight of Potatoes: *in vivo* and *in vitro* evaluation of microbial antagonists against tuber blight

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ABSTRACT

The cost of losses and control measures attributed to late blight of potatoes caused by *Phytophthora infestans*, are estimated to exceed $5 billion annually. Breeding for resistance is difficult owing to the tetraploid genotype of potato and current strains of the pathogen have developed resistance to chemical control. Consequently the search for biological control has assumed greater importance. In this investigation an *in vivo* bioassay was used to select soils antagonistic to late blight of potatoes, caused by *Phytophthora infestans.* Four out of eight samples demonstrated reproducible antagonism as determined by a reduction in the volume of tuber tissue rotted. A total of 292 bacterial and yeast samples and 20 fungal samples were recovered from suppressive soils using a variety of non-selective and selective media. When these organisms were tested individually against *P. infestans* in the assay, 15 isolates suppressed tuber rotting by >85% in at least three out of four assays. The antagonists were characterised as *Pseudomonas* spp. (3 strains), *Enterobacter* spp. (4 strains), *Bacillus* spp. (1 strain), *Pantoea* spp. (2 strains), *Citrobacter* spp. (1 strain), *Buttiauxella* spp. (1 strain), *Trichosporon* spp. (2 strains) and *Geotrichum* spp. (1 strain) by routine bacteriological tests, fatty acid profiling and partial sequencing of the gene encoding 16S or 18S (where appropriate) ribosomal RNA.

Subsequently the possible mechanisms by which the potential biocontrol agents inhibited the disease were examined. Nine isolates showed some evidence of antibiotic production with a *Pantoea* spp. producing a compound that caused the hyphae of *P. infestans* to kink and permanently cease growth. Three isolates colonised hyphae of the pathogen and eleven produced siderophores in liquid culture. Hydrogen cyanide, proteolytic, cellulolytic and beta-1,3-glucanase activity was also evident in some species.

Significant promotion of axenically grown tomato seedlings, as determined by increased stem and main root elongation, was achieved by ten of the isolates. Three population levels of the isolates were retested for disease inhibition at the end of the investigation. Isolates 3, 7 and 14 exhibited the highest levels of consistent inhibition at the lowest population levels and were therefore tested in

combination. This achieved disease suppression that, at an antagonist concentration of 25 cfu/nL, was more consistent than isolate 3 alone and was over 30% greater than either isolate 7 or 14.

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter IV

Chapter V

ABBREVIATIONS

ACC	1-aminocyclopropane-1-carboxylate
ASM	Antagonistic soil mix
ATP	Adenosine 5'-triphosphate
Avr genes	Avirulence genes
BCA	Biocontrol agent
BLAST	Basic local alignment search tool
bp	Base pair
B.B.S.	*Bacillus brevis* selective medium
B.S.S.	*Bacillus subtilis* selective medium
CAS	Chrome azurol S
CD-V8	Czapek Dox-vegetable juice
cfu	Colony forming units
CV	Crystal violet
cv	Cultivar
d	Days
DAPG	2,4-diacetylphloroglucinol
cDNA	Complementary DNA
DFOM	Deferoxamine (desferrioxamine) mesylate
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EST	Expressed sequence tag
FAME	Fatty acid methyl ester
FDA	Fluorescein diacetate
GUS	ß-glucuronidase
HCN	Hydrogen cyanide
HDTMA	Hexadecyltrimethylammonium bromide
HEPES	N(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid
HR	Hypersensitive response
IAA	Indole-3-acetic acid
I	Inosine
ISR	Induced systemic resistance
KA	King's A agar
KB	King's B agar

MES	2-(N-morpholino)-ethane sulphonic acid
MLM	Minimal liquid medium
MOPS	3-(N-morpholino)-propane sulphonic acid
mRNA	Messenger RNA
MS	Murashige and Skoog basal medium
NA	Nutrient agar
NAD	ß-nicotinamide adenine dinucleotide
NCBI	National Centre for Biotechnology Information
PCR	Polymerase chain reaction
P.C.S.	*Pseudomonas cepacia* selective medium
PDA	Potato dextrose agar
PGPR	Plant growth promoting rhizobacteria
PIPES	Piperazine-N,N'-bis[2-ethanesulphonic acid]
P-R	Peptone-rosebengal
rDNA	Ribosomal DNA
R-genes	Resistance genes
rRNA	Ribosomal RNA
RNA	Ribonucleic acid
SA	Salicylic acid
SDS	Sodium dodecyl sulphate
TAE	Tris/acetate/EDTA
TE	Tris/EDTA
Tris	Tris (hydroxymethyl) methylamine
tRNA	Transfer RNA
T.S.	*Trichoderma* selective medium
TSA	Tryptic soy agar
TSB	Tryptic soy broth
USDA	United States Department of Agriculture
UV	Ultra violet
V8	Vegetable juice
V8S	V8 agar and ß-sitosterol
V8.25	V8 agar supplemented with 0.25g $FeCl_3$ g L^{-1}
V8.5	V8 agar supplemented with 0.5g $FeCl_3$ g L^{-1}
WYE	Water-yeast extract agar
YCED	Casamino acids-yeast extract-glucose agar

CHAPTER I

Introduction

1.1 The pathogen, *Phytophthora infestans*

Phytophthora infestans (Mont.) de Bary, the causal agent of late blight, is a major pathogen of potato (*Solanum tuberosum* L.) and tomato (*Lycopersicon esculentum* Mill.).

1.2 Late blight: origin and historical impact

Most scientists agree that the centre of origin of *P. infestans* is the highlands of central Mexico. This is suggested by the greater diversity of isolates within Mexico and the apparent restriction of the A2 mating type to Mexico until the early 1980s (Fry and Goodwin, 1997). However, the occurrence of endemic isolates in the South American Andes, along with durable resistance in a number of native cultivars grown there suggest a possible alternative site of origin (Abad and Abad, 1997).

The causal agent of late blight was first established after the Irish potato famine of the 1840s which brought the disease to the attention of the Western World. This disaster depopulated Ireland by 2.5 million and led to demographic effects that are still evident today. An historic specimen from the Irish famine has recently been amplified by PCR and sequenced, demonstrating that the US-1 genotype was not solely responsible for the famine, as had previously been thought (Ristaino *et al.*, 2001).

1.3 Evolution of *P. infestans*

Recently *P. infestans* has been phylogenetically reclassified from a fungus to an oomycete. The phylum Oomycota in the Stramenopila Kingdom is actually more closely related to the golden brown algae than true fungi (Ristaino and Gumpertz, 2000). Other important pathogenic oomycetes include all *Phytophthora* species

and members of the genera *Pythium* and *Peronospora* (the downy mildews). The oomycetes encompass unique eukaryotic plant pathogens that have evolved the ability to infect plants independently of true fungi. This has led, in some instances, to distinct genetic and biochemical mechanisms with the associated advantage of circumventing certain resistance mechanisms targeted to fungi evolved by plants. For example, unlike fungi, the sterol content of oomycete membranes is negligible, making them insensitive to plant saponins (Kamoun *et al.*, 1999).

1.3 The pathogen life cycle

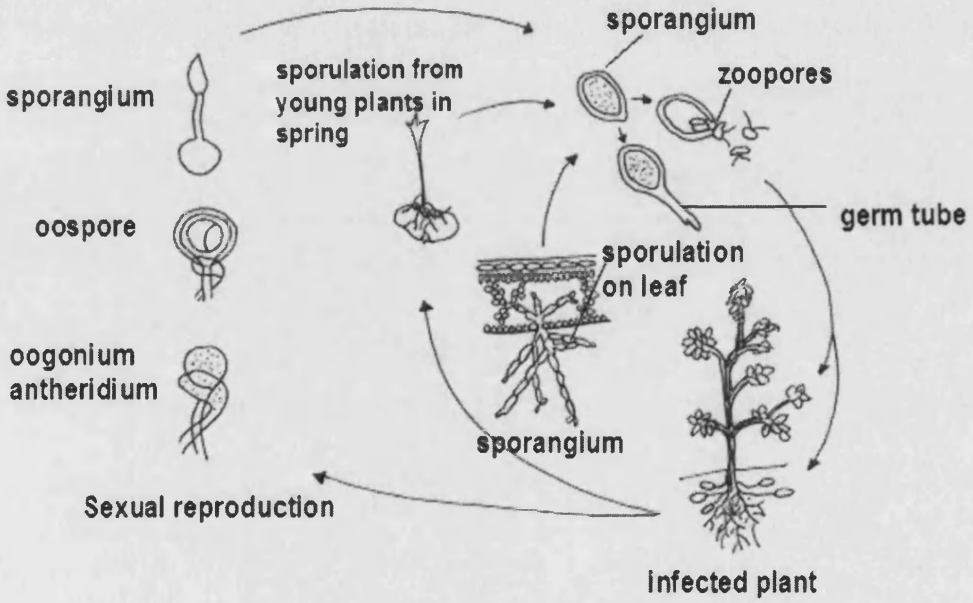

FIG. 1.1 Life cycle of *P. infestans* (adapted from Schumann, 1998). Sexual reproduction occurs only when both mating types are present.

P. infestans has a diploid life cycle but somatic cells may be polyploid (Govers, 1999). Although the organism is heterothallic, it reproduces predominantly

asexually. Two specific mating types (compatibility types), A1 and A2, are necessary for sexual reproduction. Each mating type is bisexual and capable of producing male and female gametangia that fuse with the opposite mating type and result in the formation of thick-walled oospores (Fig. 1.2, Groves and Ristaino, 2000). The oospores germinate to produce sporangia, which are wind dispersed and infectious to susceptible host plants.

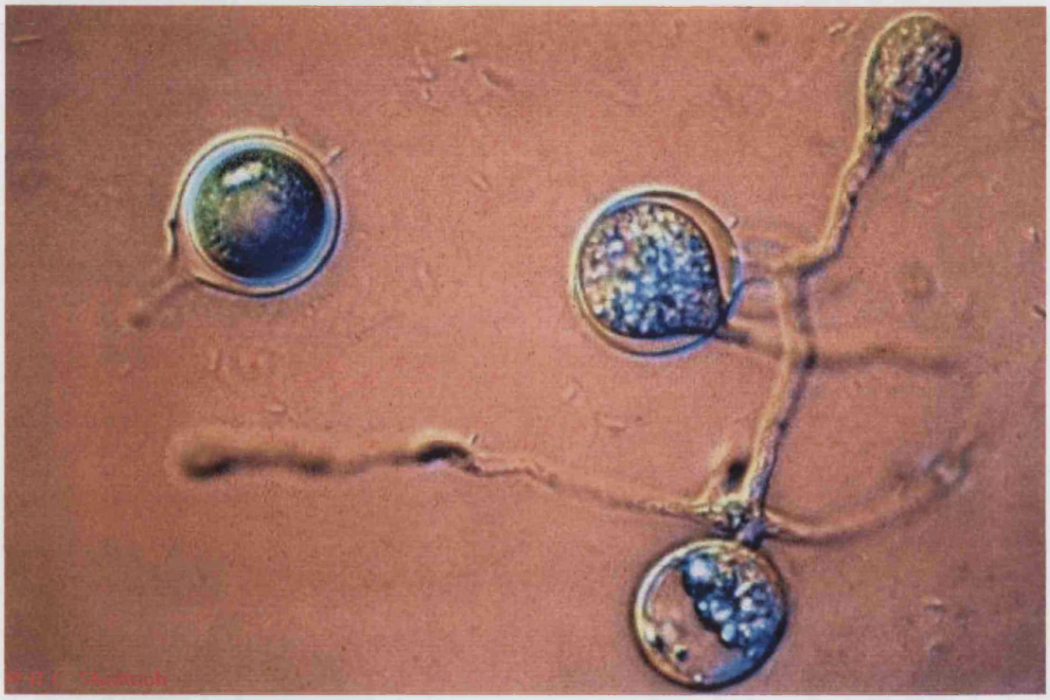

FIG. 1.2 Oospores of *P. infestans* (Shattock, 2002)

Generally infection is initiated by the release of zoospores from sporangia via the apical exit pore, although germination can occur directly by production of a germ tube. The zoospores are aquatic, lack a cell wall and have two lateral flagella, one tinsellated and the other whiplash. These swimming propagules have targeting mechanisms which play a key role in the ability of the pathogen to locate a new host during high rainfall periods (van West *et al.*, 2002). Until recently chemotaxis was thought to be the main mechanism by which zoospores were attracted to plants, however manipulation of the electrical field surrounding roots has shown that electrical signals can contribute substantially to short-range tactic responses of zoospores (van West *et al.*, 2002). On reaching the host the

zoospores encyst by a series of rapid structural changes, including the retraction or sometimes the shedding of the flagella and the formation of a cell wall. The spherical cyst attaches to solid surfaces by secretion of adhesive material and starts to germinate by a cylindrical germ tube (Krämer *et al.,* 1997). This sequence is the homing response of the zoospores with calcium playing an important part. Encystment, leading to adhesion, germination and host penetration is controlled by an autonomous calcium-mediated cascade (Deacon and Donaldson, 1993). The tip of the germ tube develops into an appressorium and this produces an infection peg which pierces the cuticle and penetrates an epidermal cell. For *P. infestans* the preferential penetration site on potato leaves is the anticlinal wall of epidermal cells immediately adjacent to stomatal guard cells (Cuypers and Hahlbrock, 1988). Inside the host cell the pathogen forms an infection vesicle. From this structure secondary hyphae germinate and grow into either mesophyll cells or the intercellular space. Further intracellular development includes formation of haustoria (Krämer *et al.,* 1997). The triggers for the formation of these infection structures in *P. infestans* are not fully understood. Krämer *et al.,* (1997) studied the synthesis of stage specific polypeptides and found that at the stage of appressoria formation, the actual start of the infection process, several major polypeptides were newly synthesised. Dramatic changes in protein content were measured when topographic features triggered differentiation, leading the authors to conclude that no additional stimulus was required.

P. infestans is a hemibiotroph having both a biotrophic and necrotrophic phase. The biotrophic phase of the disease cycle starts when an infection vesicle is produced in the epidermal cell and hyphae grow into the mesophyll cell layers both intra- and intercellularly (van West *et al.,* 1998). After three to five days the pathogen becomes necrotrophic and the hyphae spread saprophytically throughout the necrotised centre of the growing lesion before emerging through the stomata (Fig. 1.3 and 1.4).

FIG. 1.3 Potato tuber infected with *P. infestans*, exhibiting brownish shallow lesions (Fry, 2002).

FIG. 1.4 Potato foliage infected with *P. infestans,* exhibiting dark necrotic lesions and sporulation on the leaves (Mauritius Sugar Industry Research Institute, 2002).

Sporangiophores develop on the underside of the leaf and these release sporangia which propagate aerial spread of the pathogen (Birch and Whisson, 2001). Repeated asexual cycles of sporangium production and dispersal are involved in the secondary spread of the polycyclic pathogen. Sporangia or zoospores come into contact with tubers both by natural infection when rain washes spores into the soil and during harvest when tubers are lifted and come into contact with contaminated soil or infected haulms (Sato, 1979).

1.5 Molecular aspects of the infection process

The genome size of *P. infestans* is extremely large (250 megabases), twice the size of *Arabidopsis*, ten times that of yeast and much bigger than most other *Phytophthora* species (Govers, 1999). This has been a factor that has limited the understanding of the molecular processes throughout the infection process, which in turn has limited approaches to control (Beyer *et al.,* 2001). Research has primarily concentrated on the tactic responses by motile zoospores (Tyler, 2002), although differential rDNA expression during the zoosporic and germinating cyst stages has been reported in a recent paper (Paris and Lamattina, 2002). The mitochondrial rDNA relative to cytoplasmic rDNA was found to be twofold higher in zoospores and germinating cysts than in mycelia. The authors conclude that very active mitochondrial protein synthesis is evident during the zoosporic phase, followed by down-regulation of mitochondrial activity during cyst formation (Paris and Lamattina, 2002). Studies of the recognition of the pathogen have focused on pathogen substances that directly trigger a response in the host, elicitors, and on genetically identifying pathogen genes responsible for the production of such substances e.g. avirulence genes (Tyler, 2002).

A wide variety of elicitors derived from *Phytophthora* has been identified, which trigger a hypersensitive response (HR) and the activation of defence-related metabolism (Choi and Bostock, 1994). These include cell wall fragments and elicitins. The latter are restricted to *Phytophthora* and a few *Pythium* species (Ponchet *et al.,* 1999). In incompatible reactions where plants are resistant to invasion by *P. infestans,* elicitins contribute to non-host resistance. This has been demonstrated by silencing of the elicitin gene *Inf1* in *P. infestans,* which resulted in the pathogen gaining the ability to infect *Nicotiana benthamiana,* though not *N. tabacum* (Kamoun *et al.,* 1998). Recently the US Department of

Agriculture has funded a project to sequence over 50 000 expressed sequence tags (ESTs) in order to construct and maintain a *P. infestans* cDNA library (EST project, 2001). This has revealed that in addition to the class I elicitins, a diverse super-family of elicitor-like proteins is expressed (Tyler, 2002). The exact function of elicitins has not yet been elucidated, however in some cases they appear to perform a dual role as both toxins and elicitors (Strange, 2003).

DNA transformation of *P. infestans,* first achieved in the early nineties by Judelson *et al.,* (1991), has also increased understanding at the molecular level. By using a gene promoter-GUS fusion, the gene *ipiO* has been shown to be highly expressed in the hyphal tips at the edge of expanding lesions where the pathogen is invading healthy plant cells, suggesting a role in pathogenicity (van West *et al.,* 1998). Progress is, however, still slow with only 11 genes or gene families described out of an estimated 15 000 (Govers, 1999).

1.6 Mating types

Historically only the A1 mating type of *P. infestans* existed in Europe and North America. Over the last decade, however, the aggressive A2 mating type has migrated from Central or South America and has resulted in severe late blight epidemics owing to the generation of genotypes with superior fitness through sexual recombination (Judelson, 1997). The 'new' populations rapidly displaced the 'old' one, suggested to be as a result of its increased fitness over the existing 'old' clone which had been weakened by 130 years of asexual propagation (Cooke *et al.,* 2000). The severity of recent epidemics led to the Environment Protection Agency granting a section 18 emergency exemption for certain fungicides to be used in the United States (Groves and Ristaino, 2000). Additionally, sexual reproduction results in the production of oospores, which may remain infectious for up to four years depending on the substrate on which they are deposited (Turkensteen *et al.,* 2000).

1.7 Chemical control of late blight

The costs of losses and control measures attributed to late blight are estimated to exceed $5 billion annually (Birch and Whisson, 2001), making it the single most costly biotic constraint to global food production (EST project, 2001). Current measures for late blight control consist mainly of pesticide application in

conjunction with computerised disease forecasting programmes such as Blitecast and Simcast. They determine how many sprays are needed during a growing season as a function of the weather. The total amount of agricultural chemicals applied annually on potato is greater than for any other food crop. Over 300 million metric tons of potatoes are grown annually and 97% of this is treated with fungicides (Groves and Ristaino, 2000). This chemical application is both massively expensive to farmers and environmentally undesirable.

Phenylamide fungicides, such as metalaxyl, were introduced in the late 1970s and are widely used to combat diseases caused by oomycetes. However, resistance to metalaxyl, the most widely used therapeutic fungicide was found in European populations of the pathogen during the early 1980s (Fry and Goodwin, 1997). The high selection pressure applied as a result of the overuse of metalaxyl caused an increasing prevalence of resistant pathogen phenotypes in the field. In the USA, these resistant phenotypes have been found to be more aggressive than their metalaxyl-sensitive counterparts, with superior fitness as measured by reduction in latent period and increases in rate of lesion expansion, lesion area and sporulation capacity (Miller and Johnson, 2000). As a result of the fitter phenotype, the resistant genotype can be expected to persist, becoming more prevalent in the environment. This has not, however, been observed in resistant populations within the UK. In addition, metalaxyl as well as several other pesticides applied annually to potato and tomato, induce formation of the highly resistant oospores (Groves and Ristaino, 2000), although whether such oospores are viable is not clear.

1.8 Organic control

Cultural practices involving placing barriers between the crop and pathogen have had little effect in reducing late blight. For example, mulching with a range of five treatments provided only partial protection and large hill sizes, purported to protect tubers from blight by filtering spores out of the soil water suspension before reaching the plant, were ineffective (Glass *et al.,* 2001). Analysis of *P. infestans* during natural potato late blight epidemics in the Netherlands indicated that during favourable weather conditions infested organic potato fields became major inoculum sources, resulting in the spread of *P. infestans* to adjacent fields of conventionally grown potatoes (Zwankhuizen *et al.,* 1998). Clearly more efficient control measures are required for organic agriculture.

1.9 Resistant potato cultivars

Introgression of genes for resistance began at the beginning of the 20th century by crossing wild potatoes with susceptible domestic potato varieties. Germplasm was collected from the centres of origin and diversity of potatoes, Mexico, Central and South America. In total over 200 wild and 8 cultivated tuber-bearing *Solanum* spp. were sampled and taxonomically described. However, only a small number of these species have actually been used as a source of resistance genes because of the introduction of undesirable 'wild' traits with the resistant trait. Several generations of backcrossing and recurrent selection are often necessary before commercially acceptable cultivars are obtained. These difficulties are compounded by the tetraploid nature of potato which makes genetic analysis and therefore breeding particularly difficult (Gebhardt and Valkonen, 2001). Complete, durable resistance to *P. infestans* has never been found and there has been no increase in the mean level of tuber resistance in over a decade (Swiezynski and Zimnoch-Guzowska, 2001). Commercially popular cultivars such as King Edward are extremely blight susceptible.

Vertical resistance of potatoes to late blight depends on the presence of specific resistance dominant alleles (R-genes) in the host which demonstrate Mendelian inheritance. These R genes are present in a tetraploid plant in one of four allelic states (RRRR), (RRRr), (RRrr) or (Rrrr) (Gebhardt and Valkonen, 2001). This form of resistance has been largely discredited for long-term disease control because races of *P. infestans* evolve to overcome any combination of R-genes. The pathogen exists as a number of physiological races (complex races), each of which can overcome a particular combination of host R-genes. Single dominant avirulence (Avr) genes are responsible for whether a particular R gene will be effective against a given isolate. Generally an R gene is specific for a given Avr gene and therefore R genes and Avr genes have gene for gene specificity. This is proposed to result from recognition of a pathogen molecule encoded by an Avr gene by a receptor encoded by an R gene (Tyler, 2002). Recognition between these two products is required for elicitation of the defence response controlled by a wide variety of different genes and an incompatible reaction (Gebhardt and Valkonen, 2001). Absence of either the R gene or the Avr gene results in disease (a compatible reaction) (Montesano *et al.*, 2003).

It has been largely accepted that in incompatible reactions the virulence alleles are recessive to dominant avirulence alleles, with virulent races of late blight evolving by means of mutation of avirulence alleles which become ineffective in eliciting defence responses. Most genetic studies support the recessive virulence alleles / dominant avirulence alleles theory, however some unexpected segregation ratios have also been obtained. These ratios suggest additional phenomena may be influencing the inheritance of virulence. These possibilities include 1) a lower aggressiveness in a proportion of the progeny, 2) the presence of a second locus inhibiting avirulence in one parent, 3) a different locus in each parent determining avirulence/virulence on one R-gene and 4) dominance of some alleles determining virulence (Alkherb *et al.,* 1995). At least 11 R genes in potato and 2 in tomato have been described that protect against *P. infestans* infection and Avr genes corresponding to 6 R genes have been genetically defined and placed on a genetic map of the pathogen (Tyler, 2001).

Recently a major resistance gene *RB* from *Solanum bulbocastanum* was successfully cloned and expressed in potato. The transgenic plants expressed wide spectrum late blight resistance (Song *et al.*, 2003). The use of single or combinations of a few major genes is still likely, however, to lead to resistance within the pathogen population. This approach, combined with others, such as biocontrol or fungicide application may be more successful.

Non-Mendelian, quantitative variation of resistance levels, known as horizontal resistance, also exists. It is considered to be more durable than vertical resistance and is thought, in some circles, to offer the only potential long-term solution to blight control (Lowe and Harrison, 1995). This horizontal or field resistance is considered to be controlled by polygenes or minor gene complexes and does not confer immunity but slows down blight progress (Gebhardt and Valkonen, 2001). Vleeshouwers *et al.,* (2000a) examined the hypersensitive response (HR) in wild *Solanum* species, in potato cultivars carrying known R genes and in non-hosts. The effectiveness of the HR in restricting growth of the pathogen differed considerably between clones and correlated with resistance levels, leading them to suggest a possible involvement of 'weak' R gene-Avr gene interactions in this partial resistance. In partially or field resistant clones some lesion development did occur, with the growth rate of the lesions differing between clones indicating that defence mechanisms other than HR are also

operating. Further research has indicated that constitutive expression of pathogenesis-related genes may also contribute to non-specific resistance to *P. infestans* in *Solanum* (Vleeshouwers *et al.,* 2000b).

The lack of understanding of genetic determination of both vertical and horizontal resistance severely hinders the development of durable resistant cultivars. In addition to this the phenotypic expression of tuber resistance depends to a large extent on testing conditions with data from different countries often differing significantly (Swiezynski and Zimnich-Guzowska, 2001).

Two recent technical developments should contribute substantially to the development of potato cultivars with increasing resistance levels. The manipulation of the ploidy level in the host, with the ability to reduce tetraploid to diploid and the associated simplification of genetic analysis. In addition to this has been the expanding use of DNA-based genetic markers, which has allowed the construction of molecular linkage maps that produce the framework for the location of loci for pathogen resistance in the potato genome (Gebhardt and Valkonen, 2001).

1.10 The *P. infestans* EST project

There has been limited direct genomic sequencing of *P. infestans*, owing both to its large genome and to a relative lack of public funding (Walton, 2001). However the previously mentioned EST project should greatly facilitate genetic methodologies and have a huge impact on research ranging from fungicide development to population dynamics (EST project, 2001). In addition to this the advent of DNA fingerprinting is proving invaluable for providing information on epidemiology, population structure, variability within populations, survival in natural conditions and infection sources (Bakonyi *et al.,* 2002, Zwankhuizen *et al.,* 1998). Recently a survey of 61 940 ESTs generated for potato plant aerial tissue, below-ground tissue and tissues challenged with *P. infestans* was undertaken. Cluster analysis allowed identification of a number of characterised as well as novel sequences, unique to the late blight – potato interaction, including defence and resistance related genes and genes implicated in signal transduction and gene regulation. This may aid understanding of the mechanism of resistance (Ronning *et al.,* 2003).

1.11 Biocontrol: the advantages

The current limitations of control measures have increased the necessity of finding other means to control late blight. Biological control has two particularly significant advantages over chemical control, the obvious environmental benefits of reduced pollution and increased longevity of the protectant (Emmert and Handelsman, 1999). There are considerable incentives for industry to develop biologically based alternatives to chemicals. Regulatory requirements are less stringent for a biological than a chemical pesticide and this in turn provides a quicker market entry and therefore return on investment. A possible limitation is, however, the length of time for development of the biocontrol agent (BCA), which can be considerable. Public opinion is also in favour of biological pest control as opposed to chemical (Froyd, 1997).

In the past, widespread success with biological control agents of plant diseases has been limited, largely owing to the unpredictable effects of edaphic, climatic and agronomic conditions on the microorganism(s). However, substantial developments with microbial techniques and an increasing awareness of the important ecological parameters which govern the efficacy of biological control are improving this. As a result, the number of commercially produced agents are increasing.

1.12 Suppressive soils

The literature documents a number of cases where soils, as a result of their microbial make-up, are sources of suppression for soilborne plant diseases. The Chateaurenard soils in France and the Salinas Valley soil in California are well known for their natural suppressiveness to Fusarium wilt diseases. Nonpathogenic *Fusarium oxysporum* and fluorescent *Pseudomonas* spp. are mainly responsible for this suppression, operating by mechanisms such as competition and induced systemic resistance (Lemanceau *et al.,* 1993; Duijff *et al.,* 1998). The most extensively studied of suppressive soils are those antagonistic to take-all of wheat caused by *Gaeumannomyces graminis* var. *tritici*

which occur worldwide. Weller and co-workers (2002) have characterised the activity for these soils in Washington State, showing fluorescent *Pseudomonas* spp. to be responsible. These bacteria build up in the suppressive soils and produce the antifungal metabolite 2,4-diacetylphloroglucinol (DAPG). Soils exhibiting suppression to late blight have been found in Brittany, France. All three soils studied showed lysis of up to 60% of *P. infestans* sporangia. One particularly suppressive soil was characterised by low germination rates and intense lysis of sporangia of the pathogen (Andrivon, 1994). Whether this could be attributed to abiotic or biotic factors was not established.

1.13 Biocontrol using rhizobacteria

There are two types of suppression, general and specific. The former, also called non-specific antagonism and biological buffering, refers to activity attributable to the total microbial biomass in soil and is not transferable. The latter is owing to the effects of individuals or select groups of microorganisms and is transferable (Weller *et al.,* 2002). Microorganisms formulated as biocontrol agents are isolated from soils exhibiting specific suppression because of the need to be transferable. A number of commercial formulations are available which have been isolated from the rhizosphere. For example, *Trichoderma virens*, the active ingredient of SoilGard, is registered for control of damping-off and root rots of ornamental and food crop plants (USDA, 2000). Others have been patented (Kurze *et al.,* 2001) and a number are being investigated at laboratory level (for example, Goel *et al.,* 2002). As part of a biological control program Kirk and McBeath (1998) examined the control of late blight by applying *Trichoderma atroviride* and synthetic fungicides. Emergence of plants from seed pieces inoculated with *P. infestans* which were then treated with formulations of *T. atroviride* or mancozeb were comparable to untreated and non-inoculated controls in controlled environment and field experiments. However field experiments conducted under conditions conducive to late blight development and where inoculation was aerial showed that none of the seed treatments delayed the onset and severity of late blight infection. Jindal *et al.,* (1988) found six phylloplane fungi antagonistic to *P. infestans.* Maximum control (93%) occurred when spores of *Penicillium aurantiogriseum* were applied 12h prior to inoculation. Culture filtrates of the test organisms applied simultaneously with *P. infestans* or 12h earlier also significantly reduced disease severity. Cao and Forrer (2001) reported on a number of biocontrol bacteria effective against late

blight, including a strain of *Pseudomonas putida.* However, it has not been possible to find any further information concerning this.

1.14 Why tuber not foliage?

Although late blight is commonly known for its devastation of potato foliage, controlling it at the tuber level has several advantages. Soil is an important reservoir of infection and spatial mapping of disease symptom classes has shown that the planting of infected tubers results in focal expansion of foliage disease (Ristaino and Gumpertz, 2000). Tubers are the marketable portion of the plant and therefore infection at this level is important. In addition, even when *P. infestans* infection of foliage occurs too late in the season to cause reductions in yield, sporulation on leaves when there is high rainfall can result in high levels of tuber infection. Symptom development and secondary bacterial infection can then cause rotting underground. Latent infection of potato tubers has been clearly detected by means of immunological and molecular-biological assays. Investigation of seed potato showed that up to 20% of symptomless tubers may be infected with *P. infestans,* resulting in substantial infection of progeny plants (Appel *et al.,* 2001). Severe storage losses can occur after tubers infected with *P. infestans* are held for processing at temperatures in excess of 7°C (Kirk *et al.,* 1997). In addition to these points, successful biocontrol in the phyllosphere has encountered even more problems than in the rhizosphere (Fokkema, 1993). For these reasons potential biocontrol microorganisms were selected from the rhizosphere and soil in this investigation.

1.15 Biocontrol of late blight

The lack of any adequate control measures at present increases the potential of biological control as a solution to the late blight problem (Cao and Forrer, 2001). Integration with other alternative methods of control, such as field resistant potato cultivars, may result in additive or synergistic effects that provide a satisfactory level of plant protection (Janisiewicz and Korsten, 2002). In addition it may be possible to select a consortium of antagonistic microorganisms that operate by different modes of action. This would provide less variable control and would also avoid placing a single selection pressure on the pathogen population therefore increasing longevity of the protectant.

1.16 Aims

This investigation has the following objectives: 1. to screen a variety of soils for suppression of late blight: 2. to isolate microorganisms from suppressive soils and test them individually in a tuber bioassay: 3. to identify the suppressive organisms by conventional bacteriological techniques, fatty acid profiling and partial sequencing of the 16S or 18S rRNA gene: 4. to investigate the mode of action by which the microorganisms suppress late blight: 5. to determine a consortium of two or three antagonists with additive or synergistic disease control.

CHAPTER II

Detection of antagonists, their isolation and confirmation of their antagonism

2.1 INTRODUCTION

This project is based on the transferability of specific suppression (Weller *et al.*, 2002). By selecting soils exhibiting specific suppression of late blight it was hoped that microorganisms responsible for all or some of the suppression could be isolated and identified. They could then be tested for their efficacy as biocontrol agents (BCAs).

In addition to the Brittany soils suppressive to late blight (Andrivon, 1994), there have been two preliminary studies indicating the potential for biological control of *P. infestans*, using soil as the source of suppression. Clulow *et al.*, (1995) demonstrated that tubers grown in wet compost were more resistant to late blight than those grown in dry compost. They proposed that this may be due to a greater number bacteria colonising the tuber surface in the wet compost and demonstrated direct *in vitro* antagonism by randomly selected tuber surface bacteria. Thompson (1999) screened 3 soil types using an adaptation of the method of Schisler and Slininger (1997). One soil type was identified as significantly antagonistic to tuber infection by *P. infestans.* Ten potential antagonists were isolated from the soil, grown in pure culture and applied in suspensions of all ten and in two component groups of five (after Fukui *et al.*, 1999a) to tubers which had been inoculated 2 days earlier with *P. infestans.* The mixture of ten isolates reduced disease in tubers infected with a metalaxyl resistant strain by up to 37% (p= 0.001). In many cases antagonistic plate assays showed significant inhibition of *P. infestans* by individual microorganisms.

Tentative identification indicated that antagonists included *Pseudomonas* spp. and *Bacillus* spp.

The most prominent theme in recent biological control literature is the requirement for suitable selection procedures for BCAs. Antagonists selected must be able to operate efficiently in the environmental conditions where the disease exists (Cook, 1993, Deacon, 1991). In this investigation the potato tuber bioassay rather than an *in vitro* assay was used as the latter is frequently cited as being unrepresentative of the environment and therefore the most common reason for inconsistent and inadequate control by candidate agents of disease suppression (Dickie and Bell, 1995).

The strain of *P. infestans* used in the investigation was resistant to the fungicide metalaxyl. Maintenance conditions are extremely important for the growth and development of *P. infestans,* water potential being perhaps the most significant factor influencing sporangium production of *Phytophthora* spp.. In general, relative humidity approaching 100% or matric potentials approaching zero are highly conducive to sporangium formation. Sporangia form at a range of temperatures (3 to 26°C) but the optimum is between 18 and 22°C (Ribeiro, 1983).

2.2 MATERIALS AND METHODS

2.2.1 *Phytophthora infestans* isolates

The metalaxyl resistant strain; 95.18.3.2. (obtained courtesy of Dr R. Shattock and D. Earnshaw of Bangor University, Wales) was used in all experiments.

2.2.1.1 Culture, inoculum preparation and storage

Growth of *P. infestans* was determined on V8 (30%) agar, V8 agar + β-sitosterol (V8S), V8 supplemented with $FeCl_3$ (0.5g L^{-1} and 0.25g L^{-1}) (V8.5 and V8.25), V8 (20%, 10%, 5%) and Rye A agar at 15°C. When *P. infestans* was transferred from leaflets the media contained antibiotics (rifamycin and ampicillin) (details in appendix I). Cultures of *P. infestans* were maintained on 5% V8 and all of the above media apart from Rye A were used for growth depending on the experiment. Plates were stored sealed which maintained high relative humidity levels. To maintain pathogen vigour the isolates were passed through the host after every ten sub-cultures.

Mycelial growth, sporangia production and zoospore production were measured for the above isolate on the various media. After two weeks of growth an agar plug (size 3 cork borer) containing mycelium was placed in the centre of a Petri dish and incubated at 15°C. The diameter of the colony was measured 7 and 14 days after inoculation. After 14 days the plates were flooded with 10ml sterile distilled water and the mycelium scraped with a sterile glass rod to release sporangia. The sporangial suspension was transferred to a sterile tube and the concentration of sporangia determined using a haemocytometer. In order to release zoospores, the sporangial suspension was cooled to between 4°C and 6°C for 2h and the resulting zoospore concentration counted using a haemocytometer (Medina and Platt, 1999).

Transfers of the pathogen on artificial media were minimised as this leads to a loss of pathogenicity or sporulation or both (Dhingra and Sinclair, 1995).

2.2.1.2 Inoculation and testing of pathogenicity

Zoospore suspensions were used to increase the number of inoculum units. Inoculum was prepared as above using sporangial suspensions from 2 week old

V8 or V8S cultures. Zoospore concentration was adjusted to 5 x 10^4/ml with the aid of a haemocytometer before inoculation.

Leaflet cultures were also used as a quicker method to provide inoculum. Detached leaflets of the cultivar King Edward were used for pathogenicity testing. Leaflets were obtained from plants grown from seed potatoes (Thompson & Morgan Ltd, Poplar Lane, Ipswich, England). Prior to inoculation the leaflets were washed in 2% sodium hypochlorite, then washed with 70% ethanol and finally rinsed three times with deionised water. Leaflets were placed, abaxial-side uppermost, in glass Petri dishes with damp paper towels and inoculated with zoospores (100µl of 5 x 10^4/ml). Humidity was maintained by sealing the dishes and the infected leaflets were incubated at between 18 and 20°C with a day/night light cycle of 14/10 hours. Pathogenicity was assessed based on the presence or absence of sporulating lesions.

2.2.2 Bioassay for *in vivo* selection of suppressive soils

2.2.2.1 Soil Material

Eight live soil samples were collected, including six from the rhizosphere of potatoes growing in plots and fields since effective antagonists are often found associated with the specific crop plant (Cook 1993). It was also important to select microorganisms from the environment in which they would later be required to operate. Soils were collected in Autumn 2000 and stored in plastic bags at 18 to 20°C for up to 4 months before use (see Table 2.1 for provenance, history and characteristics.)

TABLE 2.1 Provenance, history and characteristics of the eight soils screened for suppressiveness

Soil	Provenance	Previous year's history	Characteristics
1	Chippenham, Wiltshire	Potatoes grown, no blight	Sandy
2	Kent	Potatoes grown, no blight	Coarse-silty
3	Madrid, Spain	Potatoes grown, no blight	Clay loam
4	Madrid, Spain	Potatoes grown, no blight	Clay loam
5	Cambridge University Farm	Potatoes grown, no blight	Loam
6	Cambridge University Farm	Potatoes grown, blight present	Loam
7	Weybridge, Surrey	Potatoes not grown	Sandy loam
8	Purley, Surrey	Potatoes not grown	Calcareous loam

2.2.2.2 Bioassay

The assay used to select for antagonistic microorganisms was essentially that of Schisler and Slininger (1997) which was designed to select antagonists acting by both direct and indirect mechanisms (Fig. 2.1). The method was originally used to screen and successfully select microorganisms in soils suitable for control of Fusarium dry rot of stored potatoes. The adapted assay was used successfully by Thompson (1999) who screened 3 soil types, finding one highly inhibitory.

Antagonistic soil mixes (ASM), as described in Table 2.2, were prepared from each soil type.

TABLE 2.2 Components of Antagonistic Soil Mix (ASM)

Component	Quantity (g)
Sterile bulk loam[a]	77.5
Sterile sand	15.5
Live test soil	5
Potato periderm[b]	2

[a] bulk loam obtained by autoclaving garden soil three times at 121°C for 15 min, with intervals of 48 hours between each autoclaving. The sample was allowed to rest for over a week after autoclaving to ensure that any release of ammonia from the soil would not influence the experiment.

[b] see Appendix I

The mixture (Table 2.2) was adjusted to a moisture level of 0.2 m^3/m^3 and incubated in a sealed beaker at 18°C for 7 days (temperature and moisture level optimal for *P. infestans*). Table 2.3 shows the subsequent treatments. ASM pastes were adjusted to a moisture level of 0.35 m^3/m^3 before applying to the potato tuber. Tubers of the cultivar King Edward were used for all experiments. King Edward is blight susceptible usually given a rating of 2-3 for foliage and 3-4 for tubers on a scale of 1-10, where 1 is the most susceptible (Anon, 1999). It is also commercially popular. Entire tubers were used as the tuber disk model

system has recently been shown as not fully representative of intact tubers (Lulai, 2000).

Tubers were bought on the same day as experimentation in order to minimise age variation as far as possible. Before use, tubers were washed with 0.5% sodium hypochlorite and surface sterilised for 3 min in 70% ethanol. After washing three times in sterile deionised water they were allowed to air dry.

TABLE 2.3 Bioassay treatments to screen for suppressive activity against *P. infestans*

	Treatment (all tubers wounded on day 0)	
	Day 0	Day 2
Control 1		tubers inoculated with zoospores of *P. infestans*
Control 2		ASM[a] with no live soil applied to tubers
Control 3	ASM with no live soil inoculated with zoospores	inoculated ASM applied to tubers
Control 4	ASM with soil X[b] and no zoospores applied to tubers	
Soil X	ASM with soil X[b] inoculated with zoospores	ASM applied to tubers

[a] ASM = Antagonistic Soil Mix consisting of periderm, live field soil and sterile soil (see Fig. 2.1)

[b] Eight soils were screened, six from areas where potatoes were grown the previous year (see Table 2.1).

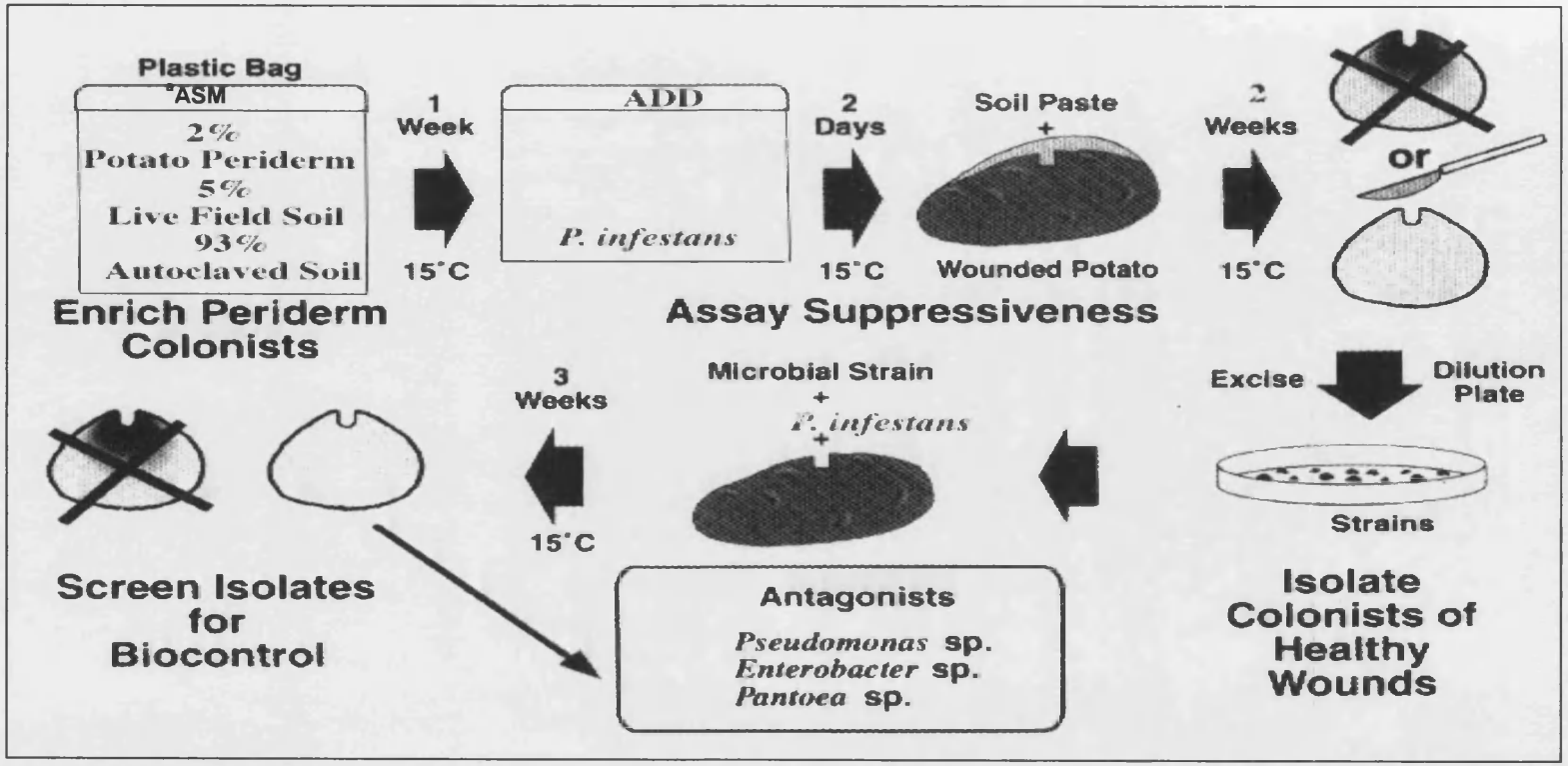

FIG. 2.1 Assay for selecting soils suppressive to late blight and for screening isolated microorganisms for antagonism to late blight, adapted from Schisler and Slininger (1997). [a]ASM = antagonistic soil mix (see Table 2.2) was incubated for 1 week at 18°C to allow proliferation of microorganisms before inoculation with *P. infestans* and incubation for a further 2 days at 18°C. The inoculated ASM and controls (see Table 2.3) were applied to tubers and incubated at 18°C. Disease was scored as lesion volume and disease index (see text) after 2 weeks. Microorganisms were isolated from wounds showing more restricted lesions than control 1 (see Table 2.3) and directly from suppressive soils. These were tested individually for antagonism in the assay.

For each treatment three plugs of tissue 8mm in diameter were removed to a depth of approximately 11mm by a sterile cork borer (size 4) from three tubers. Incubated tubers were maintained at 18°C as between 15 and 18°C is the critical temperature range necessary for infection (Sato, 1979).

After 14 days width and depth of the nine lesions were measured and used to calculate disease lesion indices (width x depth) and volumes ($V = \pi r^2 d$, where V = volume, r = radius and d = depth). In both cases the dimensions of the wound were calculated and subtracted from the measurements. Samples of inoculated tuber wounds were quartered to check the symmetry of the lesions. The assay was repeated once.

2.2.2.3 Cause of suppressiveness

Once the suppressive soils had been selected it was necessary to ascertain that the suppressive activity was caused by soil microorganisms and not to physical characteristics of the soil. This was achieved by using an assay similar to the above except that samples of each soil were autoclaved three times, rested for a week and then inoculated with *P. infestans.* Two days later the suspension was used to inoculate tubers. The control was *P. infestans* only. In order to test for physical changes, pH and soil structure were recorded before and after autoclaving.

2.2.3 Isolation of soil microorganisms

See appendix II for isolation and enrichment media

Dilutions were prepared directly from the ASMs and spread on selective and non-selective media. Colony forming unit counts were made for six days. Treatment means were calculated by averaging the Log cfu g^{-1} for each replicate.

The non-selective media used were nutrient agar (NA; Oxoid, Basingstoke, Hampshire, UK), 1/10 NA, tryptic soy agar (TSA; Oxoid), 1/10 TSA and growth medium mimicking the rhizosphere (Buyer *et al.,* 1989). The selective media were King's B (KB) medium basal formulation with the addition of novobiocin, penicillin E and cycloheximide for isolation of rhizosphere Pseudomonads (King

et al., 1954) and KB/250 with the same antibiotics. Media designed to select for *Trichoderma* spp. (T.S., Etebarian *et al.,* 2000), *Bacillus subtilis* (B.S.S., Turner and Backman, 1991), *Bacillus brevis* (B.B.S., Edwards and Seddon, 2000) and *Pseudomonas cepacia* (P.C.S., Hagedorn *et al.,* 1987) were also used. For actinomycete isolation, water-yeast extract agar (WYE) and casamino acids-yeast extract-glucose agar (YCED) were used (Crawford *et al.*, 1993); for fungi, peptone-rosebengal (P-R, Thomas, 1999) and for yeasts, yeast malt extract agar (YME; Rees, 1997).

Immediately after obtaining the assay results, microorganisms were also isolated directly from wounds of tubers which had been treated with ASMs from the four most suppressive soils. A 2mm cube of tissue was cut from around each wound and the tissue pieces macerated with a sterile scalpel. The macerate was diluted in 0.1% water agar (2 ml) and agitated for 2 minutes in a vortex mixer before making serial dilutions in 0.004% potassium phosphate buffer (pH 7.2) with 0.019% $MgCl_2$ (Schisler and Slininger, 1994).

Some dilution series were heated to 80°C for 15 min before plating, in order to select for spore producing bacteria. Dilution plates were also made from ASM samples enriched for *Pseudomonas* spp. and *Bacillus* spp.. *Bacillus* spp. endospores were enumerated by incubating ASM (2g) in 9ml Ringer solution for 10 min in a water bath at 80°C before plating. ASM (2g) was added to a tube of sodium benzoate medium (a defined, enrichment medium) and incubated for 5 days at 25°C in order to enrich for *Pseudomonas* spp. Three replicate plates were prepared for each treatment and all plates were incubated for 6 days at room temperature.

Representative organisms from all colony morphologies present were selected from the plates and streaked to purity. Isolates were stored in Microbank microvials (Pro-lab diagnostics, South Wirral, Cheshire, UK)) in liquid nitrogen. This was done according to the manufacturer's instructions; a loopful of the isolate grown for 24h on NA was aseptically added to a microvial to approximately a 3 to 4 McFarland standard. This was closed tightly and inverted 4 to 5 times shaken and then stored at -195°C in liquid nitrogen. To recover a sample of the culture the vial was opened in sterile conditions and one bead

removed. The bead was inoculated onto the required medium and the vial was returned to liquid nitrogen as quickly as possible.

2.2.4 Assay of Isolated Microorganisms

The purified isolates were tested individually against late blight using the tuber assay (Fig. 2.1). Bacterial and yeast isolates were prepared by suspending 18h cultures from solid NA medium in 10 mM potassium phosphate buffer pH 7.2 to an approximate concentration of 1 x 10^8 cfu/ml (bacterial concentrations were adjusted to an absorbance of 0.170 at 620nm and yeast concentrations were determined using a haemocytometer). Fungal isolates were prepared by growing on potato dextrose agar (PDA) with rifamycin and ampicillin for 7 days, then flooding the Petri dish with deionised water, collecting the propagule suspension and adjusting the concentration to 1 to 2 x 10^6/ml using a haemocytometer.

Tubers were prepared as for the soil test except that four wounds rather than three were made equidistant from each other, approximately 4 to 6 cm apart depending on the size of the potato tuber. Suspensions of test microorganisms and zoospores of *P. infestans* were combined in equal volumes and thoroughly mixed before inoculating the wounded potatoes (50µl per wound, corresponding to 2.5 x 10^6 cfu/wound test bacteria or yeast or 2.5 to 5 x 10^4/wound test fungi and 1.25 x 10^3/wound *P. infestans* zoospores). A different isolated test strain was used for each of the three wounds and the fourth wound served as a control (phosphate buffer and *P. infestans* only). Each isolate was tested twice. Tubers were incubated at 18°C for 3 weeks before scoring the results as disease indices and lesion volume (see section **2.2.2.2**) and expressing them as a percentage of the control. In assays where no disease developed in the control the isolate results on the same tuber were discounted. Isolates causing 85% disease control (15% disease) were re-tested at least four times and those giving at least this level of control on no fewer than three out of four occasions were designated as confirmed antagonists.

2.3 RESULTS

2.3.1 Growth of *P. infestans*

Mycelial growth, sporangia and zoospore production by *P. infestans* strain 95.18.3.2 are shown in Fig. 2.2 and 2.3.

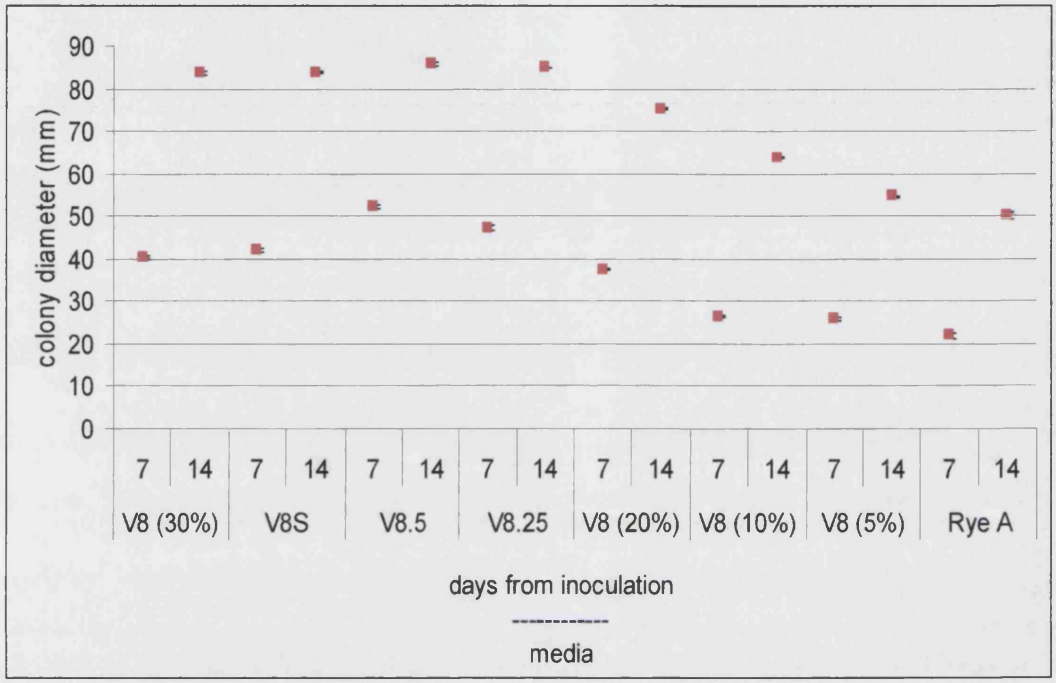

FIG. 2.2 Growth of *P. infestans* on a range of solid media. The media consist of V8 agar containing 30, 20, 10 and 5% V8 juice, V8S (30% V8 agar with ß-sitosterol), V8.5 and V8.25 (30% V8 agar with 5g and 2.5g $FeCl_3$ L^{-1} respectively) and rye A. Growth on V8.5 and V8.25 media was required for investigations in Chapter IV. Points are means of six replications with standard error bars.

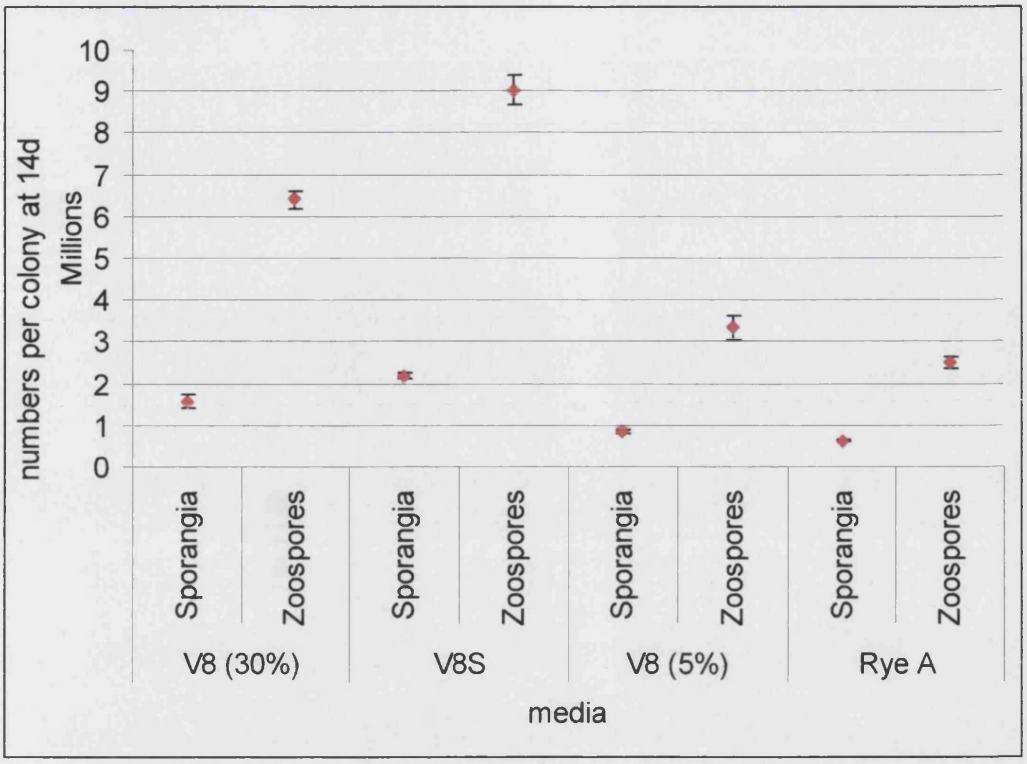

FIG. 2.3 Sporangia and zoospore production by *P. infestans* on V8 agar (30% V8 juice), V8S (30% V8 juice and ß-sitosterol), V8 agar (5% V8 juice) and Rye A. Points are means of six replications with standard error bars and represent the yield from one Petri dish colony.

The mycelial growth rates were significantly higher on V8 and V8S than Rye A and the supplement of β-sitosterol increased sporangia and zoospore production. Decreasing the concentration of V8 in the media reduced the production of infection propagules. However, the concentration of the inoculum units produced on all media was higher than that required for inoculum in the assays (5×10^4/ml propagules) (Fig. 2.3).

Growth rates on V8 supplemented with iron were significantly higher on day 7 (p = 0.001 for V8.25 and V8.5 compared to V8 (30%)). *P. infestans* maintained on 30% V8 became recalcitrant to releasing zoospores as the investigation progressed despite being passaged through the host, although it continued to release zoospores when grown on V8 agar containing only 5% V8 juice. For these reasons V8 (5%) was used throughout the investigation for culture of strain MR 95.18.3.2.

2.3.2 Bioassay of Soils

Six of the eight soils significantly reduced lesion size (Fig. 2.4). Secondary bacterial rot was frequently observed in the infected tubers in all assays (**2.3.2, 2.3.3** and **2.3.4**), which caused extensive tissue deterioration and made it difficult to delineate infection by *P. infestans*. For this reason lesions exceeding 20 000mm^3 were excluded from statistical analysis.

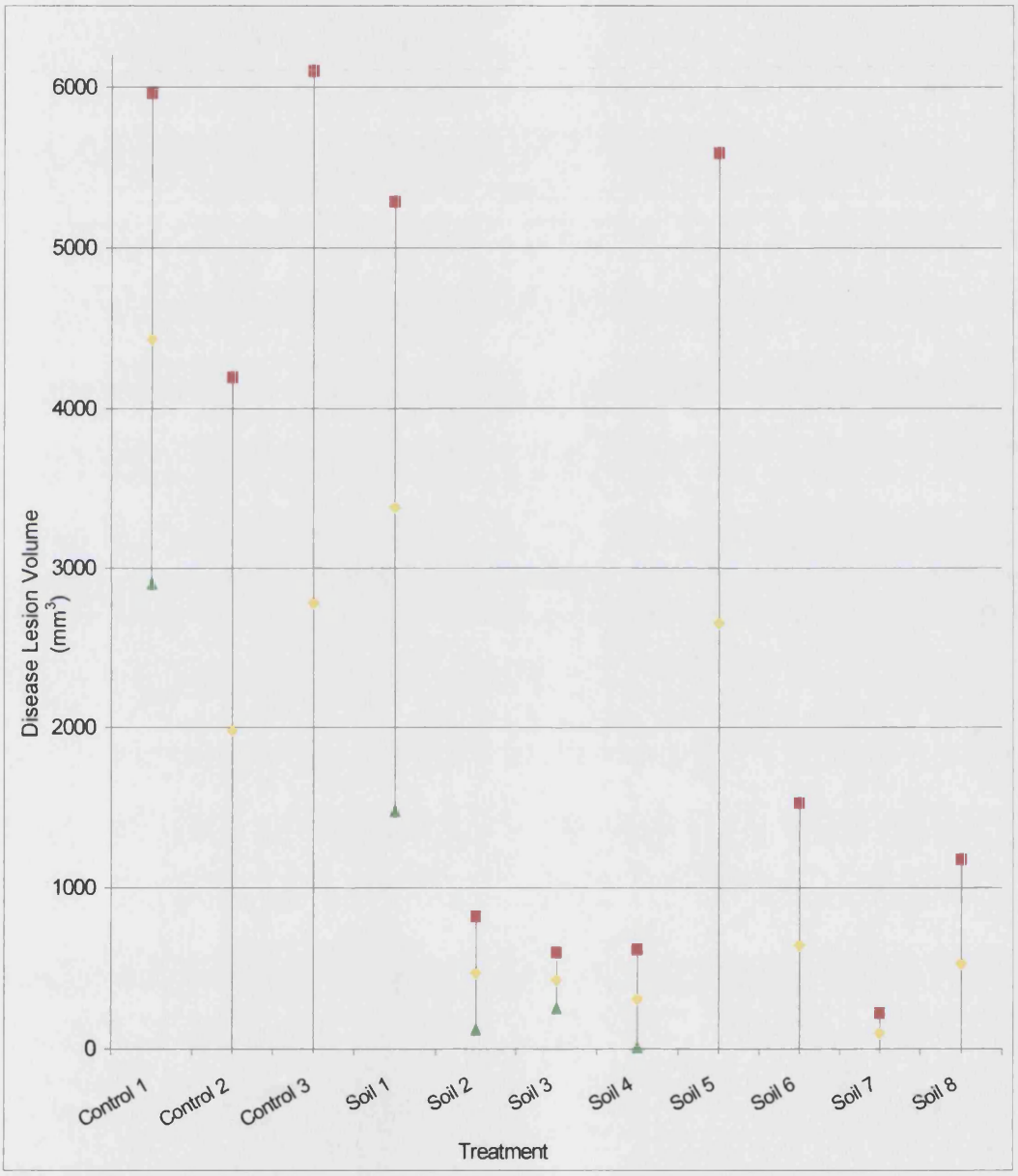

FIG. 2.4 Mean lesion volumes of *P. infestans* in tubers with 95% confidence limits after assay with antagonistic soil mixtures derived from eight soils. Control 1 is a tuber inoculated with zoospores only, control 2 is antagonistic soil mix (ASM) with no live soil applied to a tuber and control 3 is ASM with no live soil and zoospores applied to a tuber, see Table 2.3 for further details.

Repetition of the experiment gave essentially the same result (Fig. 2.5) except that suppression was less with soil 3 (mean lesion size 2863mm^3 compared with 422mm^3 for the first experiment). Lesion volumes were smaller for soils 5 and 6 and control 1 shows a much higher level of disease, while control 2 had much lower disease incidence.

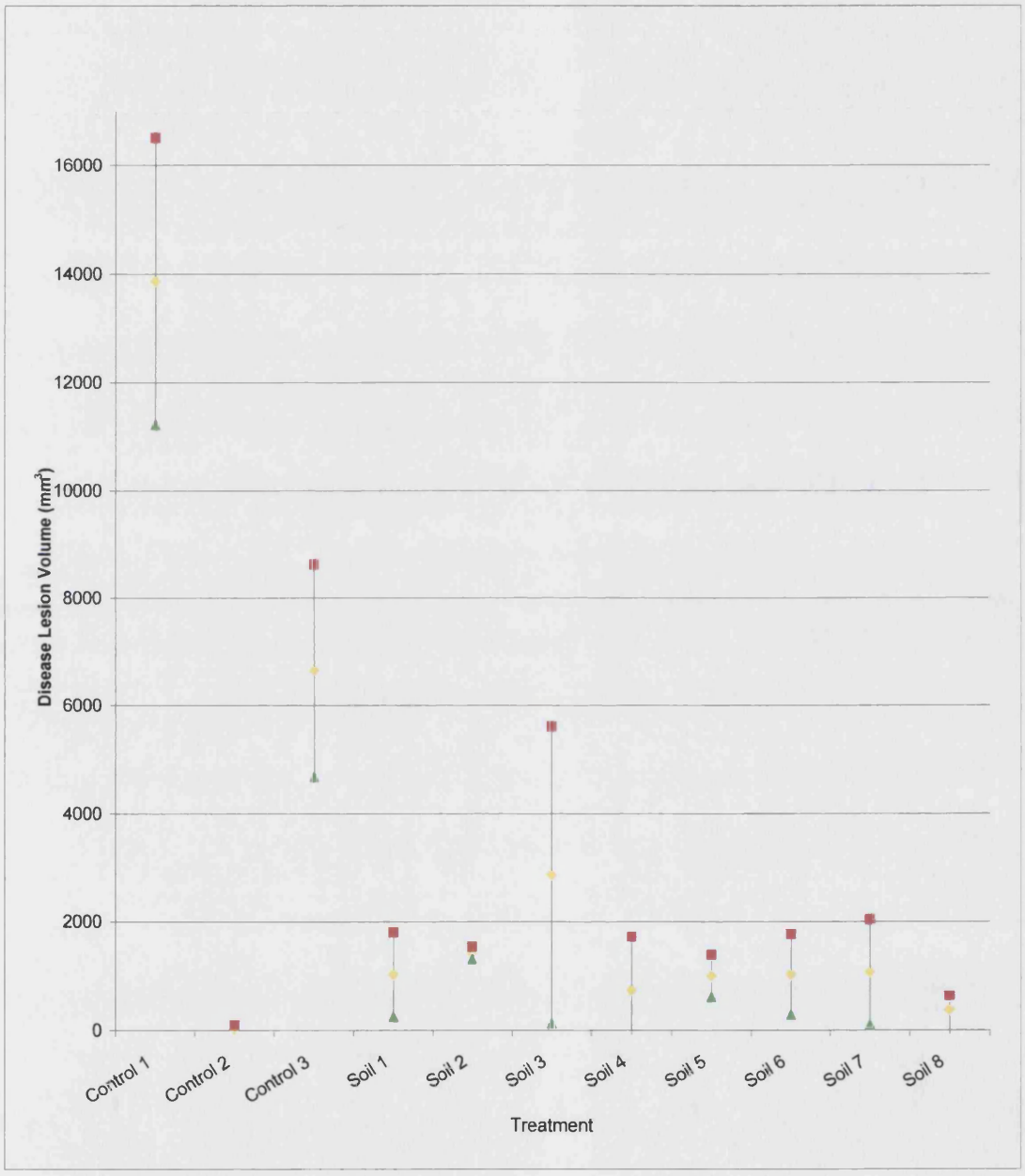

FIG. 2.5 Repetition of bioassay of soils. Mean lesion volumes of *P. infestans* in tubers with 95% confidence limits after assay with antagonistic soil mixtures derived from eight soils. For details of controls see Fig. 2.4 and Table 2.3.

2.3.3 Bioassay of test soils with no *P. infestans* (control 4)

Considerable rotting was evident when tubers were inoculated with antagonistic soil mix containing soil 5 or 6 with no *P. infestans* (control 4, see Table 2.3), the mean lesion volume being 3037mm^3 for soil 5 with a maximum of 9255mm^3 and minimum of 0mm^3 and 4839mm^3 for soil 6 with a maximum of 15784mm^3 and minimum of 0mm^3 (Fig. 2.6). Lower levels of rotting were also evident for soil 1. Lesions in these controls were attributed to soft rot bacteria which contributed to the high variation and confounded the results obtained in tests of the suppressiveness of these soils. On the basis of the bioassay results soils 2, 4, 7 and 8 were selected for further analysis.

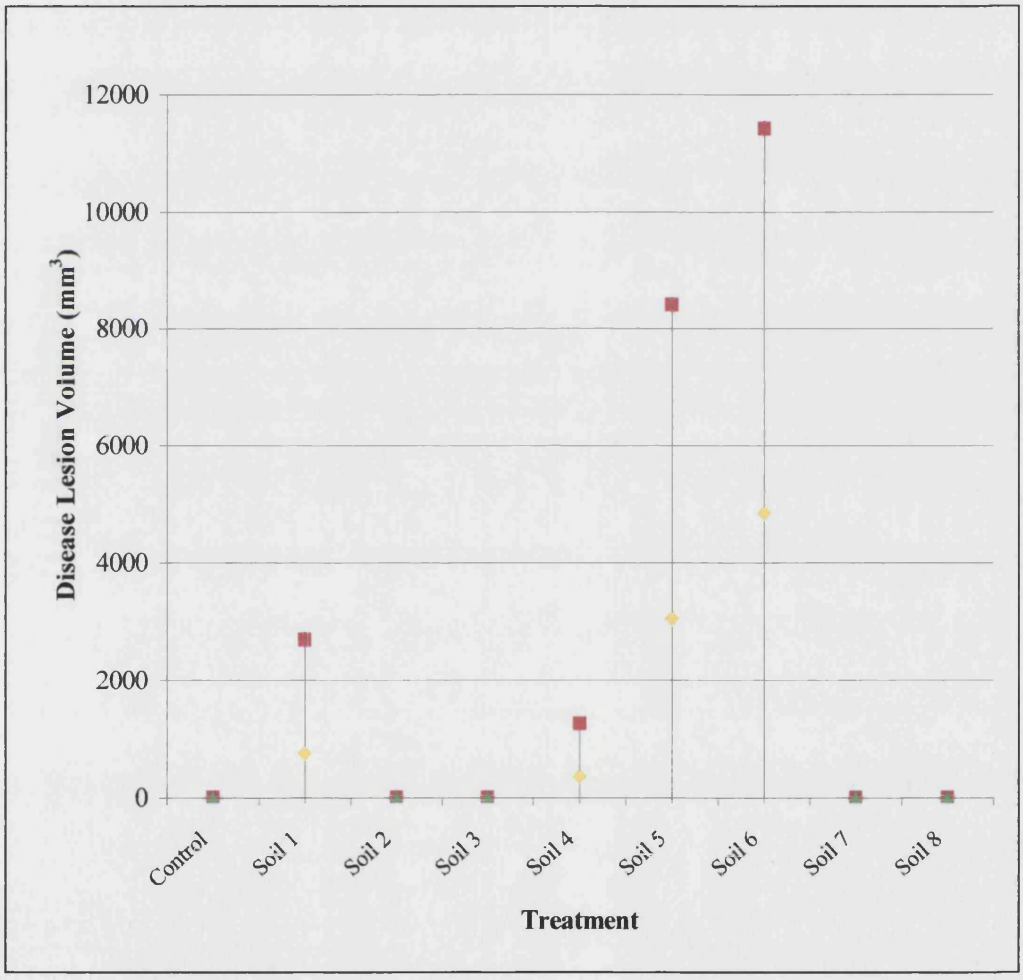

FIG. 2.6 Bioassay of control 4 (live soil tested as antagonistic soil mix in the absence of *P. infestans*) with 95% confidence limits. The control for this assay was a tuber inoculated with sterile soil.

2.3.4 Source of Suppressiveness

When soils 2, 4, 7 and 8 were autoclaved and assayed, disease symptoms were not significantly different from controls or tubers inoculated with zoospores of the pathogen although soil structure and pH remained similar to non-autoclaved samples (Fig. 2.7).

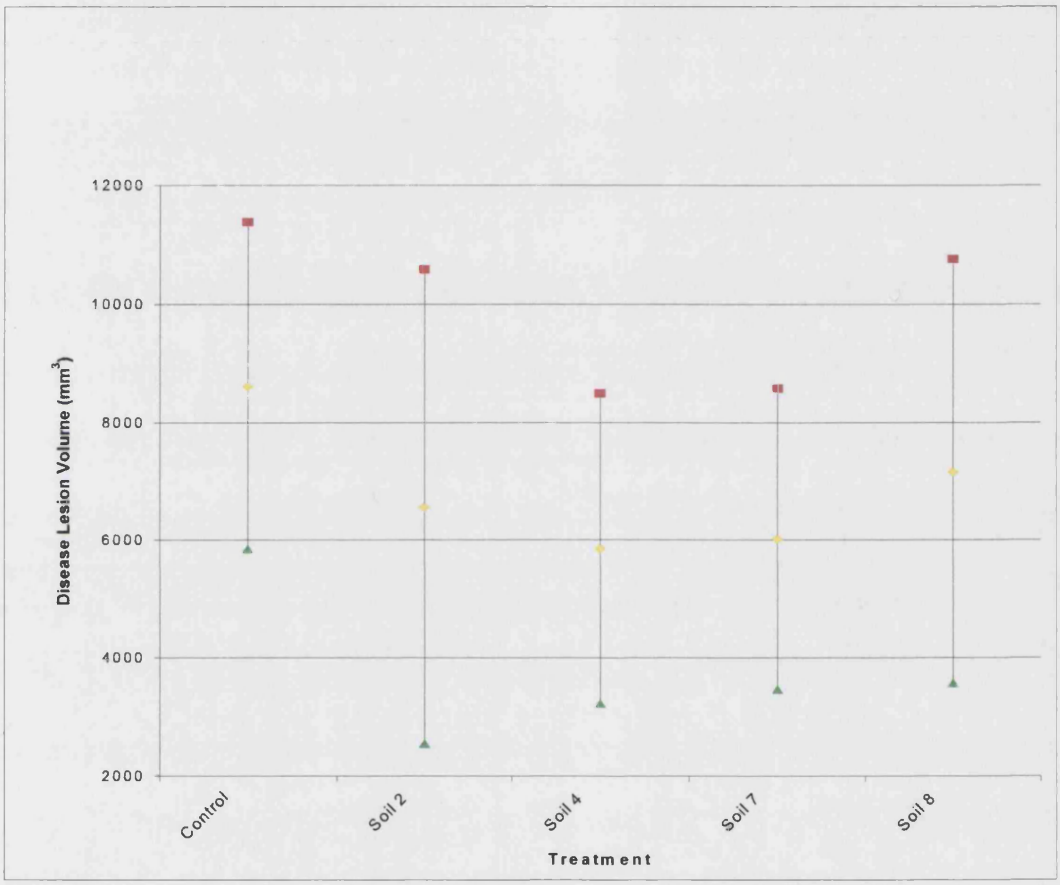

FIG. 2.7 Bioassay of autoclaved suppressive soils. Soils that had been selected as suppressive were autoclaved and retested to determine whether sterilisation would remove the source of suppressiveness. The control was a tuber inoculated with *P. infestans* only. There was no significant difference between disease lesion volume of the control and any of the autoclaved soils.

2.3.5 Isolation of microorganisms from soil samples

Data for the recovery of microbial populations from the four selected soils are summarised in Fig. 2.8.

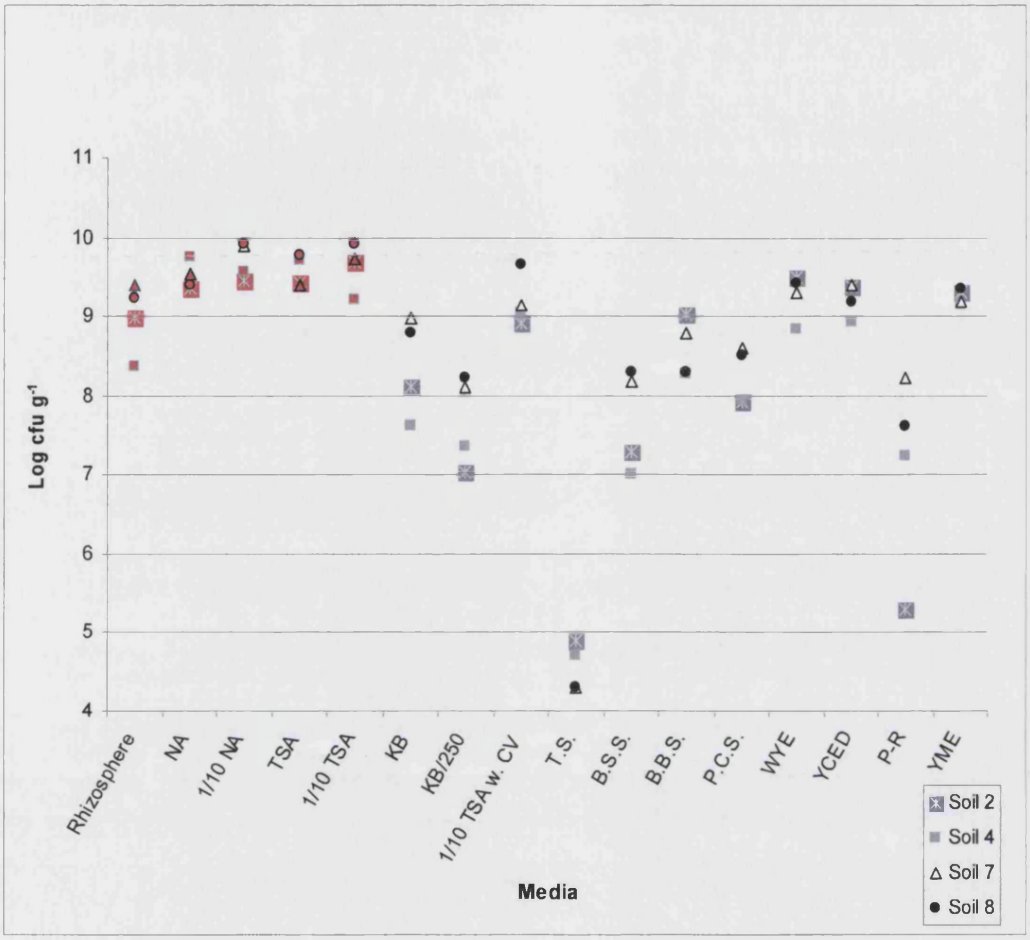

FIG. 2.8 Populations of microorganisms isolated from suppressive soils after incubation for 6 days on non-selective media (red symbols: Rhizosphere, NA, 1/10 NA, TSA and 1/10 TSA) and selective media (black symbols). For details of media see **2.2.4** and Appendix II.

Microbial recovery on non-selective media was very similar for all soils except soil 4 which was consistently lower on rhizosphere medium, the only defined non-selective medium used (Fig. 2.8). This pattern is not evident with any other soil indicating that a characteristic of the bacteria within soil 4 was responsible. Rhizosphere medium was low in iron without complex enrichments, which may have influenced the microbial isolation from soil 4. Isolation on NA required over 24h before microorganisms were evident, however recovery figures were equal or higher from day 2 than on other non-selective media (Fig. 2.9). This initial slower growth was not observed for the other soils which all had high levels of

recovery on non-selective media (data not shown). However at day one, soil 2 had a slightly lower (approximately 7.25 log cfu g^{-1}) recovery on TSA compared to the other media (approximately 7.75 to 8.25 log cfu g^{-1}).

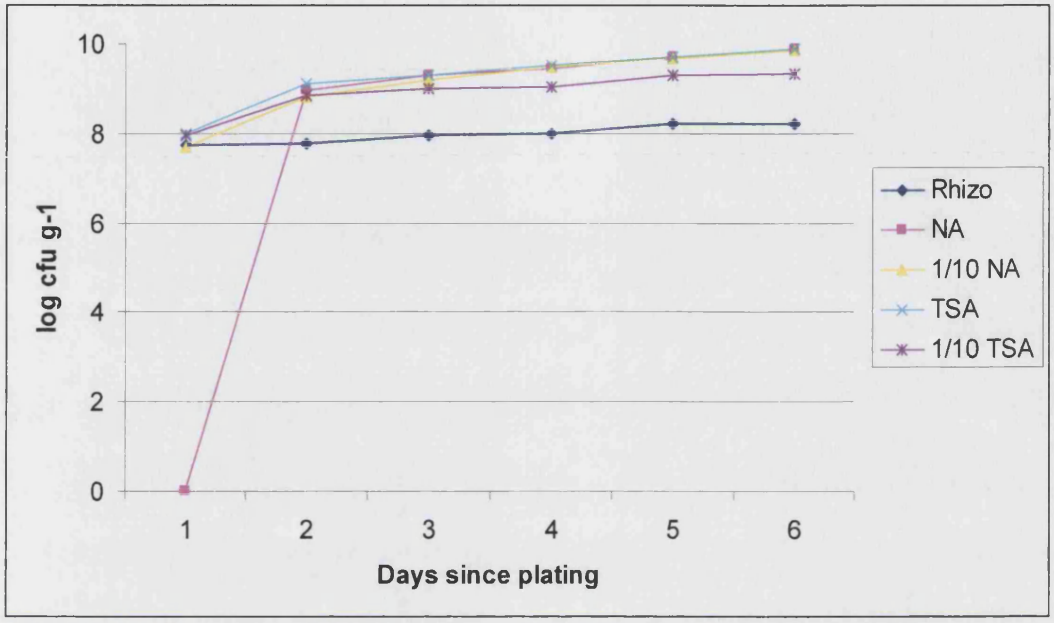

FIG. 2.9 Comparison of non-selective media for the recovery of viable bacteria from soil 4.

There was considerable variation among soils on selective media. Fluorescent pseudomonads were isolated on KB and KB/250 media but greater numbers were obtained on the full strength medium. Greater numbers of fluorescent pseudomonads were also obtained from soils 7 and 8 than soils 2 and 4. The total microbial load recovered was higher for soils 7 and 8. High levels of Gram-negative bacteria were recovered from all soils particularly soil 8, determined by isolation counts on TSA supplemented with the Gram-negative selective dye, crystal violet. The *Trichoderma* species selective medium prevented all bacterial growth and after 2 days for soils 2 and 8, 3 days for soil 4 and 5 days for soil 7, one species of fungus grew. The morphology of colonies recovered from all soils on the medium selective for *B. subtilis* corresponded to that of *B. subtilis* (irregular, undulated margin, white, dull or rough) with the highest populations being isolated from soils 7 and 8. Colony development only occurred on the medium selective for *P. cepacia* after incubation for two days, indicating suppression of most of the species that dominated the non-selective media. High

populations for all soils were isolated on WYE and YCED media, which are selective for actinomycetes, the low organic carbon content effectively controlling eubacterial and fungal growth. Aerial mycelia were present in most colonies and sometimes spores characteristic of actinomycetes but there were also high populations of colonies less characteristic of this group: these were smooth, leathery and opaque. Fungal isolation on peptone-rosebengal was similar for all soils, except soil 2 from which a significantly lower number was isolated. Recovery of yeasts on YME was similar for all soils. A selection of non-selective and selective media supporting isolated and purified bacteria is illustrated in Fig. 2.10.

FIG. 2.10 Plates from top left clockwise, peptone-rose bengal medium, *P. cepacia* selective medium, 1/10 TSA with crystal violet (purple), nutrient agar, peptone-rose bengal medium, nutrient agar and *B. subtilis* (centre).

In total 292 bacterial or yeast isolates and 20 fungal isolates were obtained from all four soils and wounds.

2.3.6 Bioassay of isolated microorganisms

There were 64 isolates that caused a reduction in lesion volume by 85% or more. After repeat testing 15 bacterial isolates suppressed disease by 85% or more on at least three out of four occasions (Fig. 2.11). Chi-squared analysis indicated that soil origin was not useful as a predictor of antagonist effectiveness.

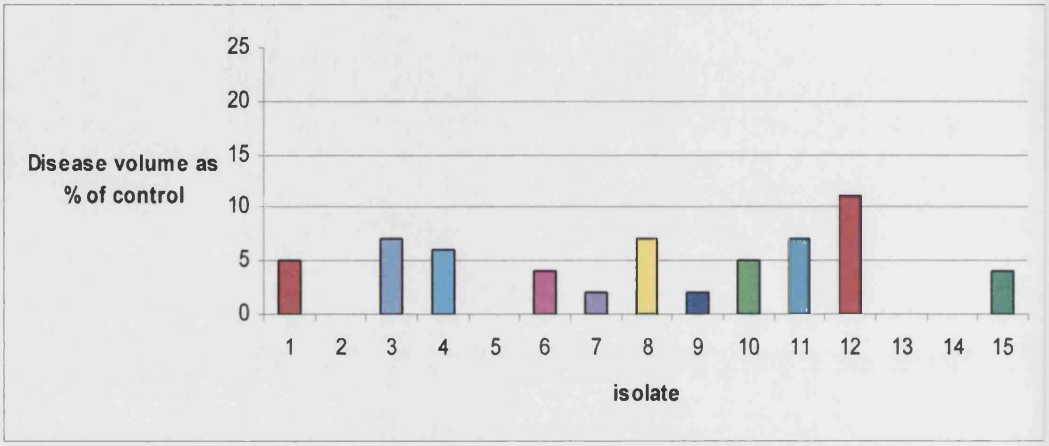

FIG. 2.11a Assay 1.

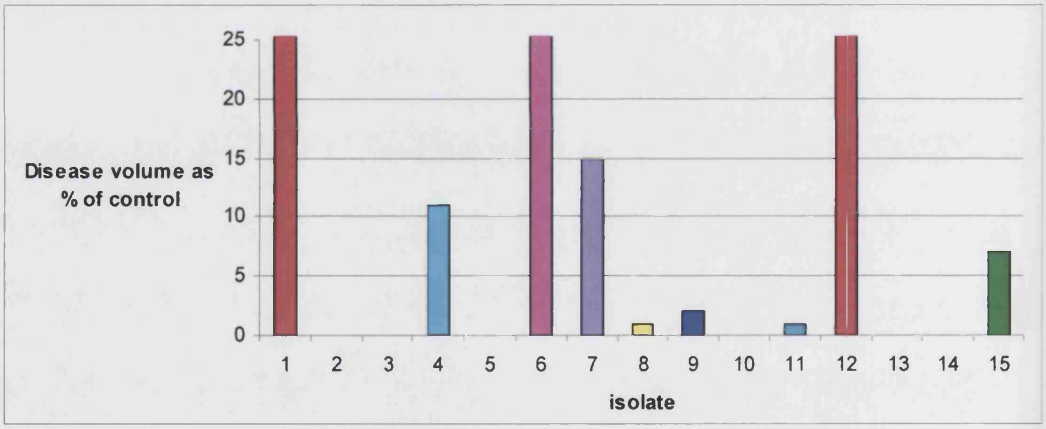

FIG. 2.11b Assay 2.

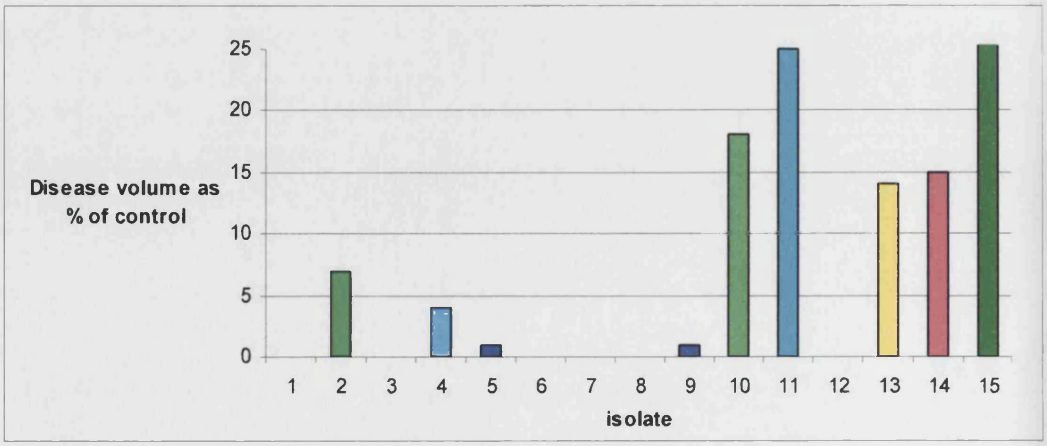

FIG. 2.11c Assay 3.

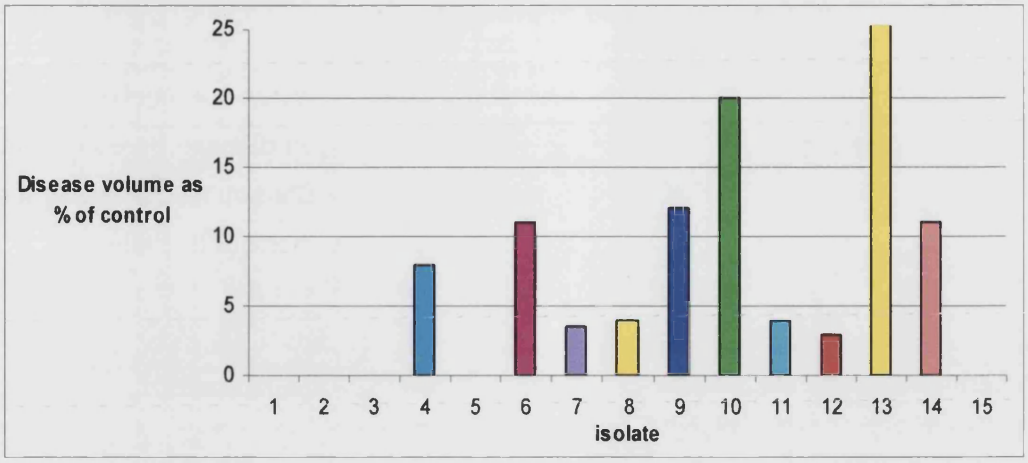

FIG. 2.11d Assay 4.

FIG. 2.11a, b, c, and d. Volumes of diseased tissue of tubers inoculated with a combination of *P. infestans* and 15 antagonists (x axis) expressed as percentages of controls inoculated with *P. infestans* only (control values ranged from 1028 – 22 317mm^3). Antagonists were assayed at least four times and all 15 reduced the volume of diseased tissue to 15% of control values or less in at least three of four trials.

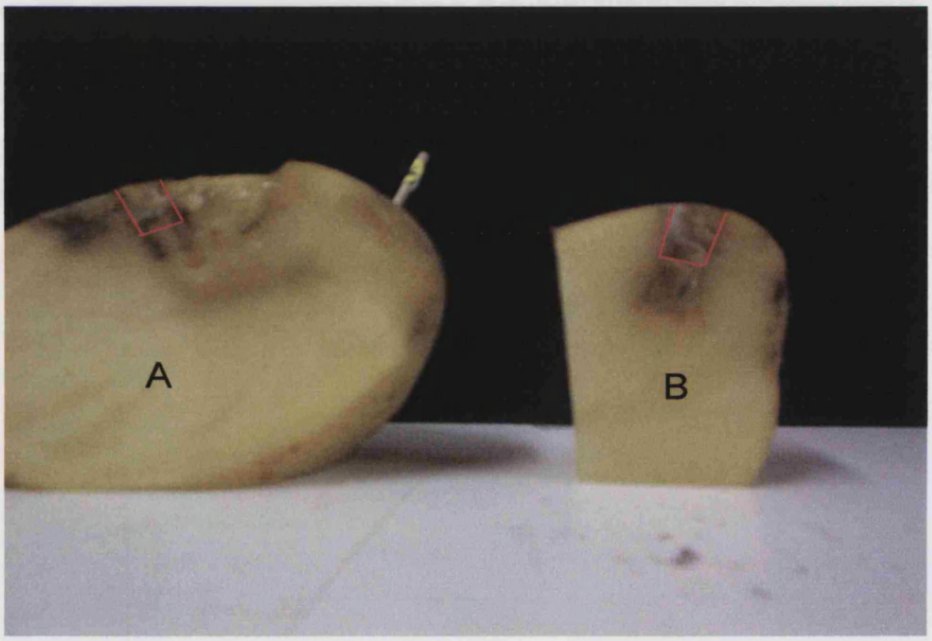

FIG. 2.12 Tuber bioassay. The control A (inoculated with *P. infestans* only) has clear disease lesions and soft rot, while B (inoculated with isolate 1) shows very limited disease development. The red lines delineate the plug wound; this tissue has darkened after contact with the atmosphere and below where the cork borer has injured tissue.

2.4 DISCUSSION

2.4.1 *Phytophthora infestans* isolates

In agreement with other workers, growth of *P. infestans* is significantly quicker on V8 based media than Rye A (Thompson, 1999) and, when supplemented with sterol, supports the highest production of sporangia and zoospores (Medina and Platt, 1999). This is expected as low concentrations of sterols are known to increase growth, stimulate asexual reproduction and influence zoospore formation (Ribeiro, 1983). V8 maintains a balance between good growth rates and sporangium production (Thompson, 1999), with oospore production being greatest when *P. infestans* is cultured on V8S (Medina and Platt, 1999). The limitation of V8 as a culture medium is that there are a significant number of *P. infestans* isolates that are V8 shy (Shaw, 1991) but this did not apply to the strain used in this investigation.

Despite passaging the pathogen through the potato host every 10 sub-cultures the organism gradually became recalcitrant to releasing zoospores when chilled to between 4°C and 6°C for over two hours. Zoospore release, however, could be achieved by reducing the concentration of V8 in the medium from 30% to 5%. The concentration of V8 media used for the culture of *Phytophthora* spp. varies throughout the literature with characterisation studies most commonly using 10% V8 agar (Fry, 2003). Those data in conjunction with the results from this investigation indicate that the optimal percentage of V8 agar for maintenance of *P. infestans* and zoospore production is between 5 and 10%. Growth rates are higher on 30% V8 and higher still on 30% V8 supplemented with Fe^{3+}; however, the high nutrient levels cause *P. infestans* to become recalcitrant to zoospore release at a faster rate than when maintained on 5% V8. This suggests that virulence would decrease at a faster rate on the higher nutrient media. The pathogen would therefore need to be passaged through the host more frequently to rebuild virulence if maintained on these media.

Iron is necessary for growth of *P. infestans* (Cuppet and Lilly, 1973), corroborated by the higher growth rate on V8 supplemented with Fe^{3+} reported in this investigation. Iron may also be important in the potato - *P. infestans* interaction as the infection of potato detached leaves by *P. infestans* was much lower when deferoxamine (desferrioxamine), an exogenous iron chelator, was added (Garcia

Mata *et al.*, 2001). These workers also demonstrated that ferritin mRNA accumulated in potato plants in response to pathogen attack.

2.4.2 Bioassay for *in vivo* selection of suppressive soils

This study adapted an assay using an entire tuber to screen for microorganisms antagonistic to *P. infestans* thus avoiding the use of antagonism screens *in vitro* which are not reflective of conditions *in vivo* (Schisler and Slininger, 1997). Moreover, the *in vivo* test allowed the selection of organisms which inhibit disease indirectly through their ability to activate host defence mechanisms.

Of the eight soils assayed, four were consistently suppressive while soils 5 and 6, which were from the same area, caused high levels of tuber decay even in the absence of *P. infestans* (control 4). Both soils had a history of potato cultivation and blight was recorded in the crop grown on soil 6 in the previous season. There was therefore the likelihood of inoculum of potato pathogens in the soil and the symptoms caused were consistent with bacterial soft rot. When the suppressive soils were autoclaved and re-assayed, soil structure and pH remained similar but antagonism to *P. infestans* was lost, strongly implicating a biological cause of suppression in the non-autoclaved samples. In agreement with Schisler and Slininger (1997) no correlation was found between soils where potatoes had been grown the year prior to sampling and suppressiveness.

High levels of variation were recorded for both soil bioassays (Figs. 2.4 and 2.5), with the soils showing variation not necessarily being consistent between the two assays. While the trend indicated by these assays was enough to select soils with suppressive characteristics and therefore for further analysis, this variation would not be acceptable when selecting isolates as potential BCAs. Unidentified organisms within the soil samples, causing secondary infections, were an important source of variation. In addition to this, potato tubers often have high numbers of bacteria species such as *Erwinia* associated with them (Morgan *et al.*, 2001). This was the main limitation to the assay as soft rot, although limited, would often affect the extent of diseased tissue. Where the tuber tissue had 'collapsed' and it was impossible to delineate late blight lesions and soft rot infections, the results were left out of the statistical analysis. Control 2, which was ASM with no live soil and no *P. infestans,* exhibited reasonably high disease incidence in the first assay but no disease incidence in the second assay. This

could be because, despite autoclaving, some residual microorganisms were still active in the soil causing rotting of the tuber in the first assay. It is also possible that microbiota associated with the potato were responsible for tuber damage.

Tuber age was an additional potential source of variation. When testing the isolated microorganisms individually against late blight, the impact of these sources of variation were minimised by ensuring that all of the same isolate results were from different tubers and that each tuber used to test the isolated microorganisms individually against *P. infestans,* was also inoculated with the control of *P. infestans* and phosphate buffer alone. The disease incidence with the test strains was then expressed as a percentage of the disease incidence of the control on the same tuber, eliminating to a large extent any bias caused by tuber characteristics.

Whole tuber assays have been used for *in vivo* screening approaches to select potential BCAs of other potato pathogens. Elson *et al.,* (1997) screened 47 agricultural soils and 7 tuber samples by spraying soil and conidial suspensions of *Helminthosporium solani,* the causal agent of potato silver scurf, onto potato tubers. From this experiment; 430 isolates of bacteria, yeasts and actinomycetes were recovered from 12 soil samples determined as suppressive. Thirteen isolates were selected for antagonistic activity including a strain of *Pseudomonas putida.* However, the authors found it difficult to obtain control on a consistent basis. Several of the isolates reduced the disease level to that of the uninoculated control in some experiments, yet variability was so high that many strains that substantially reduced disease on some occasions were not significantly different from the inoculated controls on others (Elson *et al.,* 1997). The authors conclude that determination of the mechanism of disease inhibition could explain the variability and help improve performance of the potential BCA.

Michaud *et al.,* (2002) screened 100 soils from Quebec using a similar method to that of Elson *et al.,* (1997). Of these, ten soils suppressed silver scurf development on tubers. Microorganisms were then isolated from these soils and tested individually by spraying suspensions over tubers infected as above. Efficacy of the selected microorganisms was tested at a range of temperatures to reflect the differences in temperature when potato scurf may be a problem, i.e. in the field (15 to 24°C) and when being stored (5 to 12°C). This approach would increase the potential application of the selected BCAs. However, a

disadvantage of the method is that by using whole tubers to test each microorganism the experiment is less space efficient. More significantly neither of the above investigations account for the considerable differences between disease susceptibility of tubers and this would probably have contributed substantially to variability of results.

Pseudomonas, Enterobacter and *Pantoea* spp. (Schisler and Slininger, 1997) and *Bacillus* spp. (Sadfi *et al.,* 2002) have been selected as antagonists of Fusarium dry rot of potatoes using essentially the same assay as this investigation. Sadfi *et al.,* (2002) conducted antagonism tests under greenhouse conditions. This involved immersing seed potatoes in suspensions of *Bacillus* isolates and then planting the potatoes in *Fusarium* infested soil. Field trials were also conducted with seed potatoes that were bacterised as above, immersed in a *Fusarium* conidial suspension and then planted. In addition, the antagonists were compared to the efficiency of the fungicide carbendazim to which the pathogen population is becoming resistant. Certain potential BCAs were more effective, particularly in combinations, than the fungicide at suppressing disease in the field at 40 days after planting (DAP). Most of these antagonists reduced disease compared to the control at 75 DAP but these resulted in 100% dry rot by 91 DAP in the field, which was the same for the fungicide. However, *B. cereus* reduced dry rot incidence to 0%. This demonstrates the potential of the whole tuber assay for selecting successful BCAs that may prove superior to chemical control particularly where resistance in the pathogen population is a problem. It also indicates the need to test for the efficacy of the BCAs over longer periods of time.

An alternative approach was recently used to select for bacterial biocontrol agents of the oomycetic pathogen, *Pythium aphanidermatum* in cucumber. The screening procedure was based on ecophysiological traits determined by carbon source utilisation profiles and growth rates of bacteria as indicators of a partial niche overlap with the pathogen. The bacteria were grown on a range of substrates reported to be used by the pathogen zoospores during the infection process (Folman *et al.,* 2003). This approach was unsuccessful, however, with only one isolate suppressing disease consistently indicating that initial selection based on bioassays is necessary. However, BCAs of bacterial speck of tomato (causal agent, *Pseudomonas syringae* pv. tomato) were successfully selected by similarities in carbon source utilisation profiles (Ji and Wilson, 2002).

The limited number of microorganisms that it is possible to isolate from soil and maintain in culture is well documented (Theron and Cloete, 2000). The least selective media currently used may isolate 1% to 10% of the total soil bacteria and 5% to 15% of the fungal population of soils. It is therefore possible that while having a suppressive soil and subsequently isolating microorganisms from that soil, the source of suppressiveness would not necessarily be recovered. In addition to this, the suppressiveness may be attributed to interactions between a number of organisms in the rhizosphere sample. Therefore microorganisms were isolated from the soil and from potato tuber wounds, where soils inoculated with *P. infestans* were applied to tubers but disease suppression was evident. This approach was intended to increase the proportion of microorganisms recovered with disease suppressing characteristics and efficient rhizosphere colonising abilities, both important features of an effective BCA. Enrichment of the rhizosphere samples was intended to increase the chance of recovery of antagonistic microorganisms. Many workers use this technique to increase the chance of isolating microorganisms with particular characteristics (Daane *et al.*, 2001).

Sodium benzoate medium is a commonly used substrate for enrichment of *Pseudomonas fluorescens* and some other closely related species. The only carbon source in this medium is the aromatic compound, sodium benzoate. The organisms isolated, unlike most, have the necessary enzymes to break the benzene ring and can therefore grow fairly readily in this medium, soon dominating other species introduced from the soil sample.

This investigation used a high number of non-selective and selective media to maximise recovery. The non-selective rhizosphere mimicking medium was designed for the study of siderophore-mediated interactions and the authors suggest it may be useful in screening potential BCAs of root-zone pathogens (Buyers *et al.*, 1989). Recovery of microorganisms on this medium was lower for one soil (soil 4) indicating that use for this should always be accompanied by recovery on a non-defined non-selective (NA or TSA) medium. In addition the lower recovery from this soil suggests that the medium is selective against some organisms and this may limit its use for screening potential BCAs. The lower recovery from the same soil (4) on NA on day one suggests a characteristic of the soil also influencing isolation on this media. However, this effect had

disappeared by day two suggesting the presence of spores. The slightly lower initial recovery from soil 2 on TSA, on day one, indicates that soil properties and these non-definable media may affect each other and therefore isolation rates. Ensuring that samples are incubated on isolating media for more than one day would eliminate this factor. In most cases there was little difference between NA and TSA and their dilutions indicating it would only be necessary to use one type of non-selective medium.

The effectiveness of each selective medium could not be assessed as the microorganisms were not identified. However, a range of media that provide different nutrient conditions will increase the potential for the isolation of a variety of microorganisms. In contrast to the findings of Sugimoto *et al.,* (1990) higher numbers of bacteria were recovered on full strength King's B than on the 250-fold diluted medium. Sugimoto and co-workers (1990) proposed that the bulk soil, and possibly some zones of the rhizosphere, are environments of low-nutrient availability and would contain high numbers of oligotrophic microbial communities which would be more effectively isolated by use of low nutrient media (KB/250). That this investigation found higher recovery on the full strength media suggests that the microorganisms dominating these rhizosphere samples were not oligotrophic.

The similar loads of microorganisms recorded on the Gram negative selecting TSA supplemented with crystal violet and the non-selective TSA indicate a high proportion of Gram negative bacteria. This reflects the phenomenon of Gram negative bacteria occurring in higher numbers in the rhizosphere, while Gram positive bacteria and fungi predominate in root free soil (Sugimoto *et al.,* 1990). After isolation and purification it was important to store the antagonists in liquid nitrogen, as it is a common laboratory phenomenon for bacteria to change phenotype (Barnett *et al.,* 1998).

In this investigation 292 bacterial, yeast and 20 fungal isolates were screened. However, only one fungal isolate was found to be antagonistic to *P. infestans.* This may be partly attributable to the much lower proportion recovered from the soil in comparison to bacteria. The types of selective media could be expanded to specifically select for a higher number of fungal species. Fungal species have not been used extensively for biocontrol of oomycetes although there are some successful examples, such as the control of *Phytophthora capsici* by *Trichoderma*

harzianum through the induction of systemic resistance (Ahmed *et al.*, 2000). Schisler and Slininger (1997), who exhaustively screened yeasts using the same selection techniques as this investigation, found none to be inhibitory against dry rot of stored potatoes. In this investigation, however, two yeasts that were inhibitory to *P. infestans*, were found.

CHAPTER III

Identification and Characterisation of Antagonists

3.1 INTRODUCTION

Traditional bacterial identification by substrate metabolism is being increasingly replaced by more advanced methods such as fatty acid methyl ester analysis and sequencing of genes. Identification by fatty acid profiling is based on growth and harvesting of bacteria under defined conditions, saponification of cellular lipids, methylation of fatty acids, extraction and purification of the fatty acid methyl esters (Stead *et al.,* 1992). The fatty acid methyl esters are then separated by gas chromatography and the resulting peaks identified and quantified.

Identification by sequencing of genes or sections of genes is based on the concept that there are widely conserved and divergent regions. Comparison of the nucleotide sequences of the divergent regions with sequences in online databases allows identification of isolates. Software such as the basic local alignment search tool (BLAST) and databases such as Genbank or the ribosomal database project, which contains 57 773 bacterial strain sequences, are commonly used.

The sequence of the 16S rRNA encoding gene is the most widely used for the molecular identification of, and differentiation among, bacterial species (Mollet *et al.,* 1997). This gene was the first molecule to be examined for studying prokaryotic phylogenies and was used to demonstrate the phylogenetic relationship between the eubacteria, eukaryotes and the archaebacteria. This led to all life on this planet being divided into the three domains *Bacteria, Archaea* and *Eucarya* (Woese *et al.,* 1990).

The ribosomal DNA operon in bacteria comprises three functionally and evolutionarily conserved genes, the small subunit 16S rRNA gene, the large subunit 23S rRNA gene and 5S rRNA gene, interspersed with variable spacer regions (intergenic transcribed spacer) (Fig. 3.1). Primers to conserved regions have been used to amplify ribosomal gene fragments from a broad range of phylogenetically diverse bacteria (Louws *et al.*, 1999).

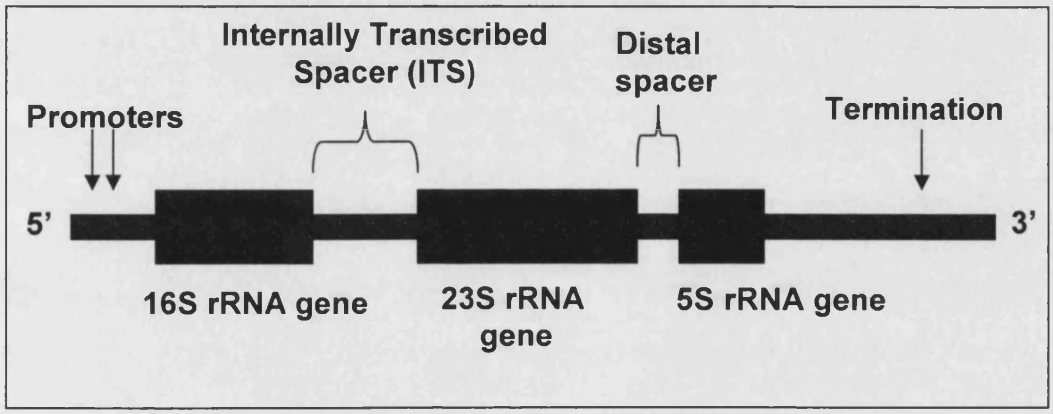

FIG. 3.1 Organisation of ribosomal DNA operons in bacteria (adapted from Louws *et al.*, 1999)

Sequencing of the 16S gene or the internally transcribed spacer region between the 16S and 23S gene are most commonly used for bacterial identification. However, caution is required with the latter if being used to differentiate or identify closely related bacterial species. This is because variation among operons within a multi-rDNA-operon genome may be as great as the variation between closely related strains (Louws *et al.*, 1999). For this reason the 16S rRNA gene was sequenced in this investigation.

Sequencing of the 18S rRNA genes is commonly used for eukaryotic identification. The eukaryotic ribosomal repeat region consists of a small subunit 18S rRNA gene, a large subunit 28S rRNA gene and the 5.8S rRNA gene also interspersed with internal transcribed regions (ITS1 and ITS2) (Fig. 3.2).

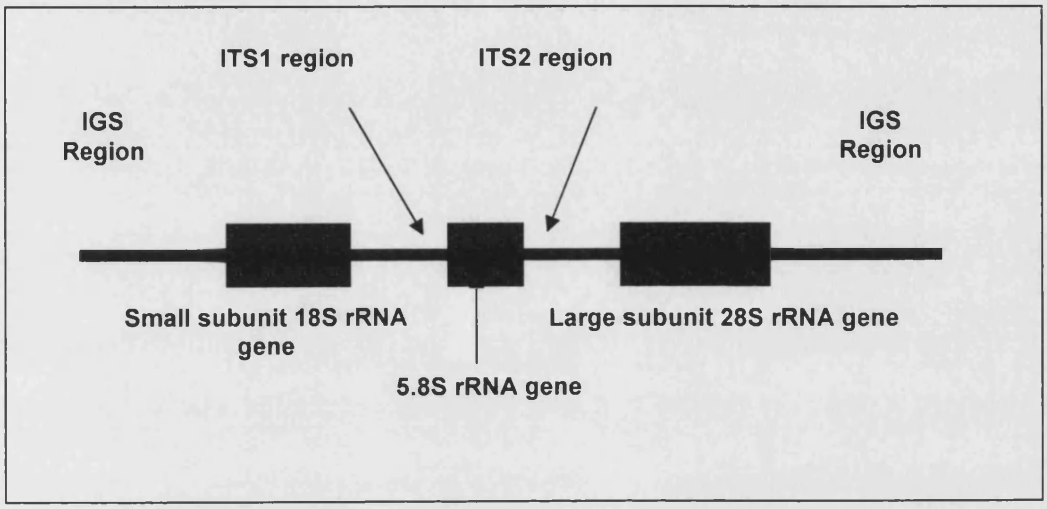

FIG. 3.2 Organisation of ribosomal DNA in eukaryotic cells.
(IGS = intergenic spacer, ITS = internally transcribed spacer)

3.2 MATERIALS AND METHODS

3.2.1 Phenotypic characterisation of isolates.

See appendix II for details of media and appendix III for phenotypic tests. Cell morphology was studied using a microscope (Olympus BH). Bacterial isolates were Gram stained and tested for catalase and oxidase activity by standard microbiological techniques (Murray *et al.,* 1994). These isolates were also checked for fluorescent pigment production by growing on King's A (KA) and KB media and evaluated visually using a UV light (Chromato-vue model CC-20, Ultra-Violet Products Inc., Cambridge, UK). All isolates were grown on YME and oatmeal agar and examined for morphology characteristic of actinomycetes (Anon, 2001).

Acid and alkaline phosphatase activity, tryptophan hydrolysis and lipid hydrolysis were measured by standard microbiological methods (Murray *et al.,* 1994). Gelatine hydrolysis, phenol oxidation and starch hydrolysis were measured by the method of Kloepper *et al.,* (1991), using the gelatine medium of Thomas (1999). Production of HCN was measured according to Kremer and Souissi (2001). Lecithinase activity was determined on egg yolk agar (Murray *et al.,* 1994) and cellulolytic and proteolytic activity by the method of Alstrom (2001).

3.2.2 Fatty Acid Methyl Ester (FAME)

FAME analysis was kindly performed by Dr David Stead of the Central Science Laboratory, York (Stead *et al.,* 1992).

3.2.3 Genotypic identification of isolates by partial sequencing and analysis of the 16S and 18S rRNA gene

3.2.3.1 DNA isolation

Bacterial DNA was extracted with the protocol adapted for bacterial DNA extraction using the Nucleon BACC1 extraction kit for blood and cells (Fry *et al.*, 2000; Tepnel Life Sciences, UK). Isolates from which DNA was successfully purified by this method but which would not amplify with a range of primers, was extracted as follows: two loopfuls of bacteria grown on NA were suspended in 1.5ml of TE buffer (10mM Tris.HCl pH 7.5, 1 mM Na_2EDTA), lysozyme was added to a final concentration of 50μg/ml and the mixture incubated at 37°C for

15 to 30 min. Lysozyme action was monitored by placing 100µl of cell suspension on a slide and adding a few drops of 10% sodium dodecyl sulphate (SDS). If the cells became clear and viscous, SDS (10%) and RNAase were added, to final concentrations of 1% and 20µg/ml respectively, to the rest of the sample and mixed by inverting before incubating at 62°C for 5-10 minutes (Malek *et al.,* 2000). Cold absolute ethanol (2.5ml) was added to precipitate the DNA, which was spooled on a glass rod and suspended in a new microfuge tube containing 200µl TE buffer.

Alternative methods used for isolates recalcitrant to extraction by either of the above methods were (1) to grow the bacteria in liquid yeast-glucose medium and add penicillin at the logarithmic growth phase, (2) pass an isolate suspension through a cell disruptor, (3) suspend a loopful in TE buffer (pH 8), boil for 15 minutes and treat with RNAase as above.

Subsequently, isolates 13, 14 and 15 were restreaked to purity 4 times and reanalysed. The DNA was extracted by picking a single colony with a sterile pipette tip and resuspending in sterile distilled water (20µl). Colony DNA was isolated by heat denaturation for 5 min at 95°C.

Samples of DNA (10µl) were electrophoresed on an agarose gel (0.5%) at 76 V for 1.5 hr containing 2µg/ml ethidium bromide with *Hin*dIII λ digest as a size marker. Gel images were taken using a whit/UV transilluminator (UVP, Inc., Cambridge, England) connected to a gel documentation system (UVP, Inc., Cambridge, England) and video graphic printer (Sony, UP-890CE).

3.2.3.2 PCR

Either 50ng of DNA or 3µl aliquot of boiled solution (where the amount of DNA could not be estimated) or 1µl of solution denatured at 95°C for 5 min was added to 0.5ml tubes containing "Ready to go" PCR beads (Amersham Pharmacia Biotech, Sweden). Each bead yields a reaction mixture containing approximately 2.5 units of *Taq* DNA polymerase, 10mM Tris-HCl (pH 9.0), 50mM KCl, 1.5mM $MgCl_2$, 200µM of each dNTP, stabilisers and BSA when made up to 25µl with sterile distilled water. Primer pairs, except EukA and EukB, (Table 3.1), were added (10pmol each) and the final volume of the reaction mixture made up to 25µl with sterile pure water. The PCR protocol began with a 5-min denaturation

step at 94°C followed by 35 cycles at 94°C for 30 sec, annealing at primer-specific temperature (Table 3.1) for 1 min and 72°C for 1 min, concluding with extension at 72°C for 5 min, using a Techne Progene Thermocycler (Jepson Bolton & Co Ltd, Watford, Herts). PCR products were separated by electrophoresis in an agarose gel (1.5% w/v agarose gel containing 2μg/ml ethidium bromide) in TAE buffer, pH 8, using a 100bp DNA ladder as a molecular size marker. Gel images were taken as before.

DNA extracted from isolates 13, 14 and 15 by denaturation at 95°C were amplified in a reaction mixture of total volume 50μl containing 1μl template DNA, 50pmol (each) primer (EukA and EukB), 200nM (each) deoxynucleotide triphosphate, 1.5mM $MgCl_2$ and 1 unit Expand High Fidelity DNA polymerase (Roche, Basel, Switzerland). PCR amplification was conducted in a PCR Sprint thermal cycler (Thermo Hybaid, Germany). The PCR program consisted of an initial denaturation step of 94°C for 3 min followed by 30 cycles of 94°C for 30s, 55°C for 30s and 72°C for 2 min, finishing with an elongation step of 72°C for 20 min.

All PCR amplifications included a negative control consisting of the reaction mixture and primers but no template.

TABLE 3.1 Sequence and characteristics of oligonucleotide primers used for amplification and partial sequencing of rRNA genes

Primer	Sequence (5'-3')	Target position[a] (group)	Annealing temperature (°C)	Reference
I[b]-341f	CCT ACG GGI GGC IGC A	341 – 356 Bacteria	Touchdown technique	Watanbe *et al.* (2001)
I-534r	TIA CCG III CTI CTG GCA C	515 – 533 Bacteria	Touchdown technique	Watanbe *et al.* (2001)
27f	AGA GTT TGA TYM TGG CTC AG[c]	27 – 47 Bacteria	55	Dees and Ghiorse (2001)
1492r	GGT TAC CTT GTT ACG ACT T	1473 – 1492 Bacteria	55	Dees and Ghiorse (2001)
341f	CTA CGG GAG GCA GCA GTG GG	341 – 361 Bacteria	55	Edwards *et al.* (1989)
928r	CCG TCA ATT CCT TTG AGT TT	908 – 928 Bacteria	55	Edwards *et al.* (1989)
1073r	ACG AGC TGA CGA CAG CCA TG	1053 – 1073 Bacteria	55	Edwards *et al.* (1989)
F243	GGA TGA GCC CGC GGC CTA	243 – 261 *Actinobacteria*[d]	63	Dees and Ghiorse (2001)
R513	CGG CCG CGG CTG CTG GCA CGT A	491 – 513 *Actinobacteria*	63	Dees and Ghiorse (2001)
P5 – for	GGT CTG AGA GGA TGA TCA GT	292 – 311 *Pseudomonas* sp.[e]	65	Stach *et al.* (2001)
P5 – rev	TTA GCT CCA CCT CGC GGC	1262 – 1280 *Pseudomonas* sp.	65	Stach *et al.* (2001)
B1	GAG CTG ACG ACA GCC ATG CAG	Sequencing	This study	This study
B2	CAC ACT GGA ACT GAG ACA C	Sequencing	This study	This study
Z5	CAA GCC TGA TGC AGC CAT G	Sequencing	This study	This study
Z6	CAT TGT AGC ACG TGT GTA GC	Sequencing	This study	This study
EukA	AACCTGGTTGATCCTGCC AGT	1 – 21 Eukarya	55	Díez *et al.*, (2001)
EukB	TGATCCTTCTGCAGGTTCA CCTAC	1795 – 1772 Eukarya	55	Díez *et al.*, (2001)

[a] positions of complementary sequence in *E. coli*

[b] I = inosine

[c] Y = C or T, M = C or A

[d] / [e] these primers were used to obtain better quality sequences from isolates identified as *Actinomycetes*[c] or *Pseudomonas* spp.[d] by preliminary sequencing using universal primers

3.2.3.3 Sequencing and analysis

PCR products were purified using QIAquick PCR purification columns (Qiagen, Crawley, UK) according to the manufacturer's instructions or were gel extracted to reduce contamination using a QIAquick gel extraction kit (Qiagen). PCR products were sequenced with the WellRed dye-labelled dideoxy-terminator cycle sequencing kit, dye-labelled terminators ddUTP, ddGTP, ddCTP, ddATP, dNTP mix solution, thermostable DNA polymerase and pyrophosphatase sequencing buffer (Beckman Coulter Inc., California, USA); sequencing was performed on a Beckman CEQ2000XL with an eight capillary automated DNA sequencing and fragment analysis system. The read length of the capillaries (DNA separation array, 33-75B) is 700bp with an accuracy of 98.5%. The sequencing was conducted by Lola Martinez, Wolfson Institute of Biomedical Research, University College London.

In two cases (isolates 4 and 15), PCR products from primers 27f and 1492r were ligated directly into the cloning vector pCR®2.1 (TA cloning kit; Invitrogen, San Diego, California). Purification, ligation and transformation were performed according to the manufacturer's instructions. Randomly picked white colonies were grown overnight in nutrient broth supplemented with 40µg/ml kanamycin and plasmid DNA extracted using the Miniprep kit (Qiagen) according to the manufacturer's instructions. DNA from selected clones was cut with restriction enzymes *Eco*R1 and *Eco*R1 and *Sau*3A1 and the products were resolved on an agarose gel (0.8%) to analyse for gene inserts. Images were taken as before. The gel resolution enabled selection of different sized products for sequencing with the M13 reverse primer and the M13 forward (-20) primer. The sequence information was aligned and from this primers (B1, B2, Z5 and Z6; Table 3.1) were designed in order to sequence the rest of the clones. Sequences were aligned using Sequencher 4.1 (Gene Codes Corporation, Michigan, USA) and the GenBank database and homologies identified using BLAST (http://www.ncbi.nlm.nih.gov/blast). Cloned sequences were aligned using MegaAlign, version 3.02 (DNASTAR Inc., Wisconsin, USA).

PCR amplicons obtained from DNA extracted from isolates 13, 14 and 15 by denaturation for 5min at 95°C were also cloned into the pCR®2.1 vector. Clones were cut with *Eco*R1 only and sequencing was conducted using the M13 reverse primer and the M13 forward primer only.

3.3 RESULTS

3.3.1 Phenotypic characterisation

The results are summarised in Table 3.2.

TABLE 3.2 Phenotypic characterisation of antagonistic isolates.

Isolate	Gram stain	Phosphatase activity	Catalase	Oxidase	HCN	Lecithinase activity	Cellulolytic activity	Proteolytic activity	Starch Hydrolysis	Lipid Hydrolysis
1	-	+	+	-	+	+[a]	-	+[b]	-	+
2	-	+	+	+	-	-	-	-	-	-
3	-	-	+	+	-	-	-	-	-	-
4	-	Weakly +	+	-	-	-	-	-	-	-
5	-	-	+	-	-	-	-	-	-	-
6	-	-	+	-	-	-	-	-	-	-
7	-	-	+	-	-	-	-	-	-	-
8	-	+	+	-	-	-	-	-	-	-
9	-	-	+	-	-	-	-	-	-	-
10	+	+	-	+	-	+	-	+[c]	+	-
11	-	-	+	-	-	-	-	-	-	-
12	-	+	+	-	-	-	+	-	-	+[d]
13	NA[e]	+	+	+	-	-	+	+	-	+
14	NA[e]	+	+	-	-	-	-	-	-	+
15	NA[e]	+	+	-	-	-	+	+ weakly	-	-

[a]Fig. 3.5, [b]Fig. 3.6a, [c]Fig. 3.6b, [d]Fig. 3.7 and [e]NA = not applicable

Isolate 1 had blue fluorescence on both KA and KB, properties commonly associated with *Pseudomonas aeruginosa* (Fig. 3.3).

FIG. 3.3 Isolate 1 fluorescing blue on KB medium under long wave UV light from the Chromato-Vue Cabinet.

Isolates 2 and 3 had green fluorescence on KA and KB which, with their inability to hydrolyse gelatine, is indicative of *P. putida* (Fig. 3.4).

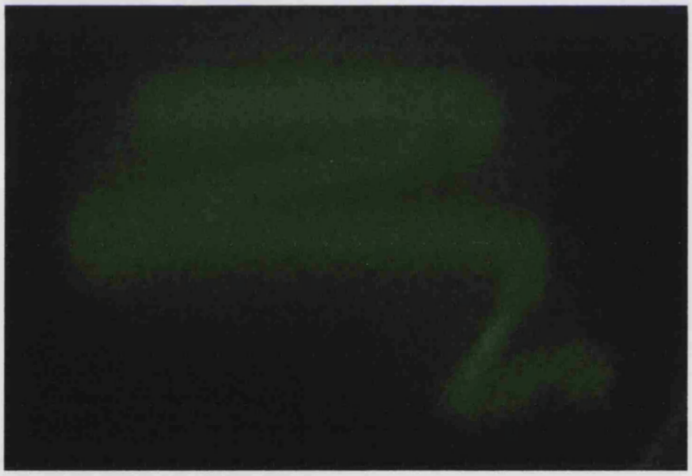

FIG. 3.4 Isolate 3 fluorescing green on KB medium under long wave UV light from the Chromato-Vue Cabinet, indicative of *P. putida*.

Phosphatase activity was recorded for 9 out of 15 isolates. The morphology of none of the isolates was indicative of the actinomycete group, *Streptomyces*. However isolates 13, 14 and 15 displayed filamentous growth into the agar on NA, YME and oatmeal agar suggestive of other actinomycetes or possibly eukaryotic organisms. All three isolates exhibited this similar growth pattern but

were clearly different from each other. None was positive for phenol oxidation or gelatine and tryptophan hydrolysis. Isolate 1 produced HCN and exhibited strong lecithinase (Fig. 3.5) and proteolytic activity (Fig. 3.6a) and lipid hydrolysis. Isolate 10 had strong proteolytic (Fig. 3.6b) and lecithinase activity and starch hydrolysis and isolates 12, 13 and 15 strong cellulolytic activity.

FIG. 3.5 Isolate 1 grown on egg yolk containing media. The lighter opaque halo surrounding the bacteria indicates lecithinase activity.

FIG. 3.6a Isolate 1.

FIG. 3.6b Isolate 10.

FIG. 3.6 Isolates grown on proteolytic media. The clear halo surrounding the bacteria indicates proteolytic activity.

Fig. 3.7 Isolate 12 demonstrating lipid hydrolysis.

FIG. 3.7 illustrates isolate 12 grown on lipid containing media. The clear halo surrounding the bacteria indicates lipase activity.

3.3.2 FAME analysis

The results are summarised in Table 3.3. This method identified 12 isolates to genus level, one further isolate to three possible genera and two isolates were unknown.

TABLE 3.3 Identification by Fatty Acid Methyl Ester (FAME) analysis.

Isolate	Match	Comment
1	*Pseudomonas* sp.	Could be *Enterobacter* sp. Seems little different from isolate 7
2	*Pseudomonas* sp.	Almost certainly *P. putida* biotype B
3	*Pseudomonas* sp.	Probably same as 7
4	Enterobacteriaceae[a]	Very close to *P. chlororaphis*
5	*Enterobacter* sp.	Possibly same as 7
6	*Enterobacter/Pantoea/Klebsiella* sp.	Probably not any species in the TSBA40 Library since closest match is <0.5[b].
7	*Enterobacter* sp.	
8	*Enterobacter* sp.	Close to some *P. putida* strains
9	*Enterobacter* sp.	Close to 3/7
10	*Bacillus* sp.	All matches were closest to *Enterobacter* spp. Values of >0.7 are often correct within this group
11	Enterobacteriaceae	Possibly same as 7.
12	*Enterobacter* sp.	
13	*Pseudomonas* sp.	All good matches & certainly close to some *Pantoea agglomerans* strains and some *Klebsiella pneumoniae* strains
14	Unknown	Possible *Enterobacter amnigenus* since S.I.[c] > 0.82. Likely to be different to 7
15	Unknown	

[a] the types of acids present, revealed by fatty acid analysis, indicate determination of genus especially in Gram negative bacteria. For these cases hydroxy acids are very important. Unfortunately, it is not always possible to determine genera within the Enterobacteriaceae

[b] comparison of the relative amounts of some fatty acids, as revealed by fatty acid analysis, with libraries of profiles gives a match as a similarity index. Values above 0.7 may be correct. Values below 0.7 are usually not correct, but this varies with genus.

[c] S.I. = similarity index

FIG. 3.8 illustrates clusters and sub-clusters as revealed by FAME analysis.

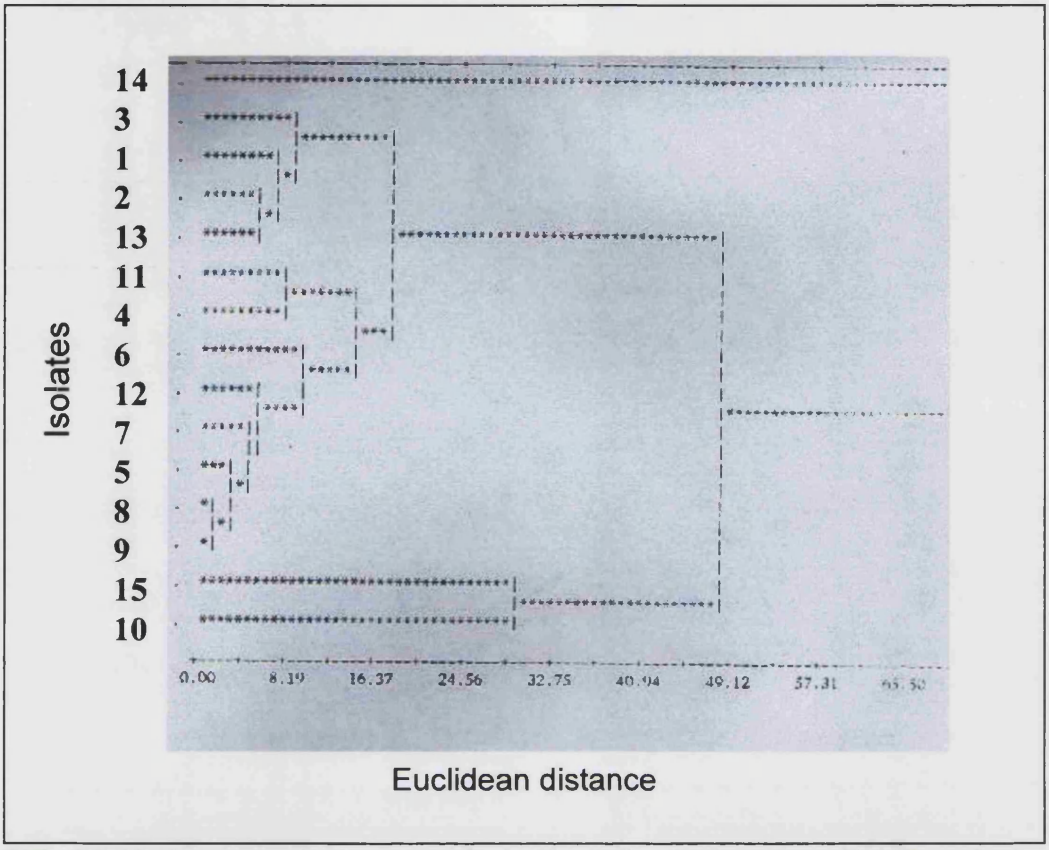

FIG. 3.8 Cluster and sub-cluster analysis of results obtained from FAME analysis (Stead, 2001). The Euclidean distance is a commonly used function, which works by treating each expression profile (in this case FAME analysis profile) as defining a point in a multidimensional space (Genomicglosseries, 2003).

3.3.3 DNA extraction, PCR amplification and sequencing

DNA was successfully extracted using the Nucleon BACC1 kit from isolates 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12 and 15. After failure to obtain an amplicon from PCR of isolates 2, 3, 7 and 8, DNA was purified using the second method. Both these methods and the addition of penicillin at the logarithmic growth phase and the use of a cell disrupter failed to extract DNA from isolates 13 and 14. Boiling samples of these isolates did not provide enough DNA to be visualised on agarose gel, but was successful for PCR.

When reanalysing isolates 13, 14 and 15, DNA was successfully extracted by heat denaturation at 95°C.

Amplicons from all target isolates and primer pairs except I-341f and I-534r were obtained. Only very faint bands were detected after visualisation on agarose gels of the products from this primer pair. Primers 27f and 1492r amplified a product of ≅ 1450bp, 341f and 928r ≅ 600bp, 27f and 1073r ≅ 1050bp, F243 and R513 ≅ 300bp and Ps–for and Ps-rev ≅ 988bp.

Direct sequencing results of some amplicons showed peaks under peaks in the raw and analysed data suggesting the presence of more than one template or different priming points. These samples were resequenced using the amplicon extracted from the agarose gel in order to reduce contamination. This was successful in some instances but sequencing with the 27f primer proved consistently difficult, the raw data suggesting multiple priming points.

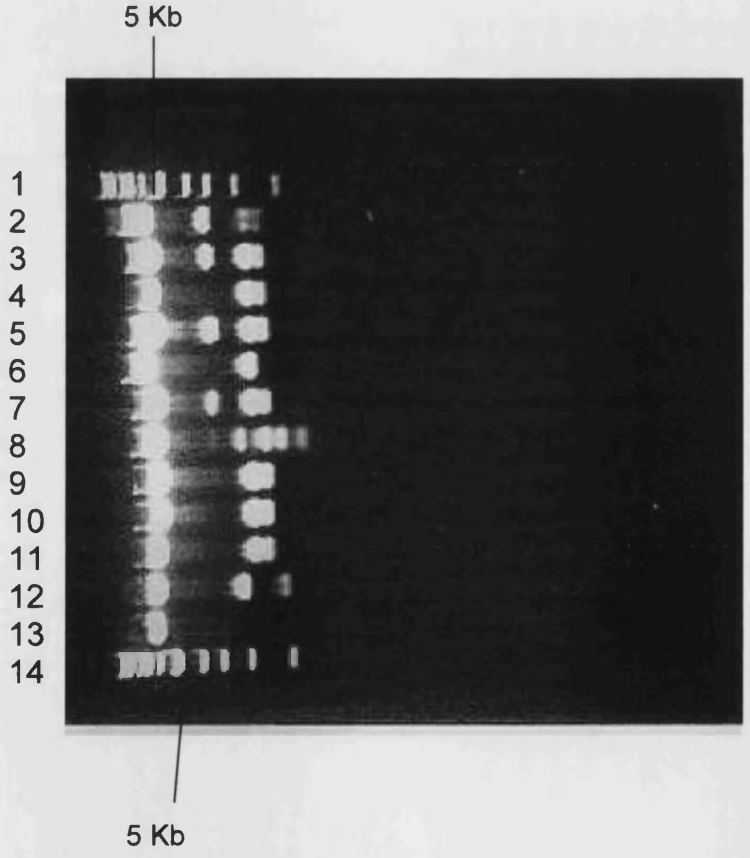

FIG. 3.9 Clone products resolved on agarose gel. Lanes 1 and 14 are 1 kb ladders (5Kb markers labelled), lanes 2 to 7 are clones of isolate 15 and lanes 8 to 13 are clones of isolate 6.

Sequencing of this section (corresponding to position 27 to ≅ 600 in *E. coli*) proved problematic for isolates 3, 4, 5, 7, 8, 9, 12, 13 and 14 with sequencing of all amplicons from 6 and 15 being particularly difficult. Amplicons from isolates 6 and 15 were therefore cloned and the products resolved by electrophoresis on agarose gels. These indicated a variety of product sizes (Fig. 3.9).

Digestion of the clones with *Eco*R1 and *Sau*3A 1 also revealed a variety of product sizes. Clones of differing sizes were selected for sequencing. The alignment of the clone sequences indicated high similarity for the 1492r sequence but was variable for the 27f, which is consistent with problems encountered in trying to sequence from base position 27. The clone sequences were aligned with a full length sequence of the species with which they were most closely identified. This allowed base position on the 16S sequence to be determined.

The sequences obtained from the primer pairs or clones were aligned in Sequencher and partial to full length sequences were obtained for all isolates (Fig. 3.10).

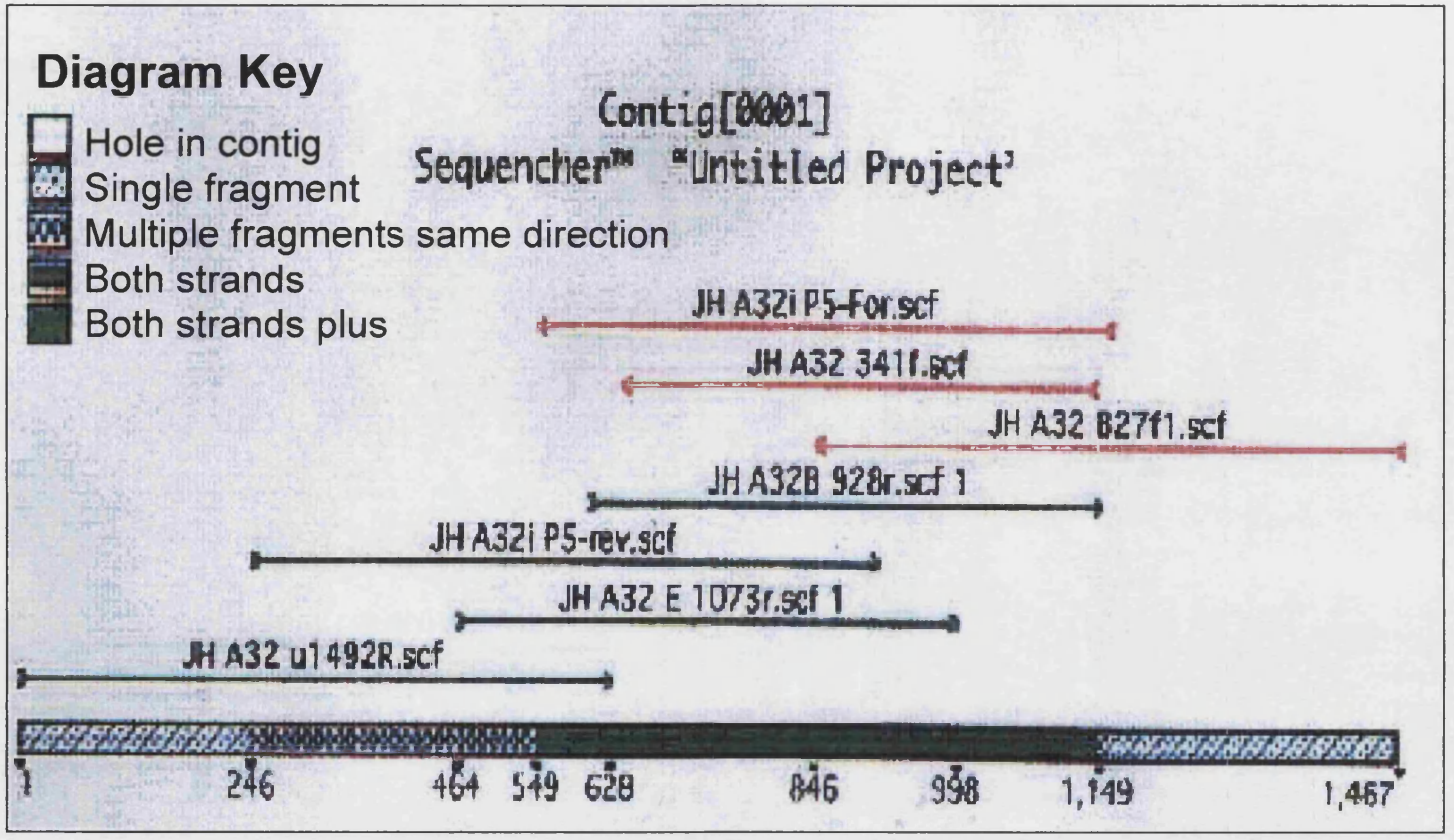

FIG. 3.10 Image from Sequencher showing alignment of different sequence segments from isolate 1 to form an almost full 16S rDNA gene sequence. JH A32 corresponds to arbitrary labelling of isolate 1 for the purpose the investigation. The subsequent labelling of each segment corresponds to the primer used to obtain that sequence. P5-for and P5-rev are the *Pseudomonas* specific primers and the others are general bacteria primers described in Table 3.1. Red arrows represent forward primed segments and black arrows reverse primed segments

The results of the BLAST enquiry are summarised in Table 3.4.

TABLE 3.4 Taxa in GenBank with 16S rRNA gene sequences most similar to those of the microorganisms isolated

Isolate	Best Match	% match	Number of bases	Reference
1	***Pseudomonas putida***	98	1422	Galdzicka *et al.*, (1998) (unpublished)[a]
2	***Arthrobacter siderocapsulatus* (a *Pseudomonas putida* strain)**	99	1185	Chun *et al.*, (2001)
3	***Pseudomonas putida* strain ATCC 17527**	99	1440	Galdzicka *et al.*, (1998) (unpublished)[a]
4	***Enterobacter amnigenus***	100	1074	Harada *et al.*, (1996)
5	***Pantoea* sp. 16S rRNA gene (agglomerans)[b]**	99	1136	Hoffmann *et al.*, (1998)
6	***Enterobacter agglomerans*[b]**	96-98[c]	610 – 711[c]	
7	***Pantoea* sp. 16S rRNA gene (*agglomerans*)[b]**	99	1118	Hoffmann *et al.*, (1998)
8	***Enterobacter agglomerans*[b]**	100	657	Rojas *et al.*, (1999) (unpublished)[a]
9	***Enterobacter* sp. 16-31**	99	558	Picard *et al.*, (2000)
10	***Bacillus thuringiensis***	100	1405	Ticknor *et al.*, (2001)
11	***Citrobacter gillenii***	99	1355	Whitney *et al.*, (1997) (unpublished)[a]
12	***Buttiauxella noackiae***	99	1113	Sproer *et al.*, (1999)
13	*Enterobacter agglomerans*[b]	98	677	Rojas *et al.*, (1999) (unpublished)[a]
13	Identification after	100	498	Sugita and Nakase,

	reanalysis[e] ***Trichosporon laibachii***			(1998)
14	*Enterobacter* sp.	99	1118	Chin *et al.*, (1995) (unpublished)[a]
14	Identification after reanalysis[e] ***Trichosporon cutaneum***	99	500	Van de Peer *et al.*, (1992)
15	*Enterobacter agglomerans*[b/d]	99	753 – 794[d]	
15	Identification after reanalysis[e] ***Galactomyces geotrichum*** Anamorph: ***Geotrichum candidum***		500	Wilmotte *et al.*, (1993)

[a] unpublished references obtained from BLAST database

[b] The species *Enterobacter agglomerans* is synonymous with *Erwinia herbicola* and is the basonym of *Pantoea agglomerans* (DSMZ, 2003)

[c] three distinct clones of various lengths were obtained from isolate 6, with all three showing closest identification to this species but at different levels of similarity (96%, 97% and 98%) and to different strains.

[d] three distinct clones of various lengths were obtained from isolate 15, with all three showing closest identification to different strains of this species. Comparison of the clones showed distinct sequence differences.

[e] isolates were reanalysed after suspected contamination

FIG. 3.11 illustrates a cluster tree of the isolates as identified by sequencing, for the purpose of comparison with the FAME analysis results.

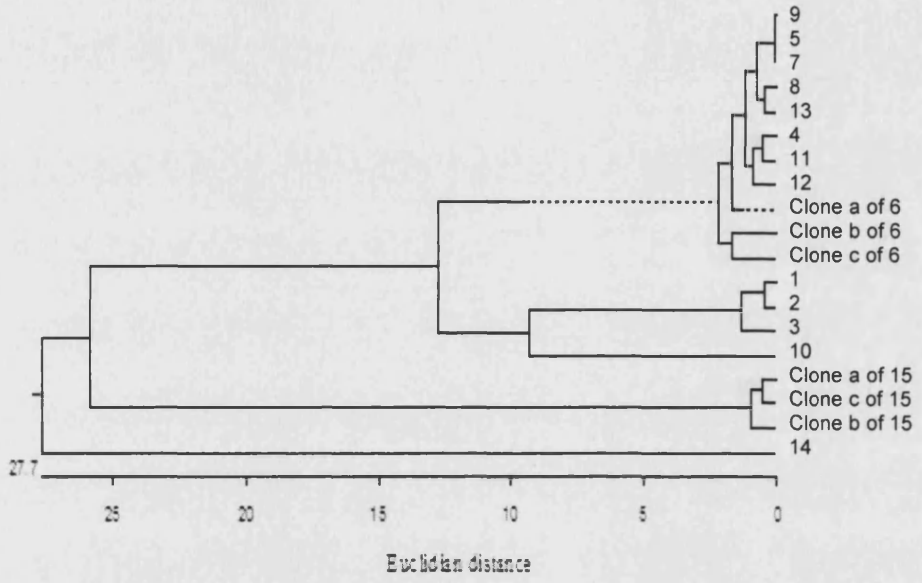

Fig. 3.11 Cluster tree of the antagonistic isolates as identified by sequencing of the 16S rRNA gene, using MegaAlign. The Euclidean distance is a commonly used function, which works by treating each expression profile (in this case 16S rDNA sequencing profile) as defining a point in a multidimensional space (Genomicglosseries, 2003). The sequences of isolates 13, 14 and 15 are the first obtained, contaminated sequences.

3.4 DISCUSSION

All 15 of the antagonists that consistently inhibited infection of potato by *P. infestans* were initially identified as bacterial. They were identified by fatty acid analysis and partial sequencing of the 16S rRNA gene. Sequencing of the 16S rRNA gene allowed identification to a similarity of 98 to 100%, in all but one case (1 clone of isolate 6). Three of the isolates (13, 14 and 15) displayed colony growth, morphology and dimensions that were inconsistent with the identified species (*Enterobacter* spp.). The colony growth was filamentous and sporulating suggesting actinomycete species, yeast or fungi. Contamination of the gene templates is extremely easy (personal communication Whiting, 2003) and the isolates were therefore reanalysed after repurification and primers designed to amplify the 18S rDNA gene were used as well as 16S primers. Positive results were obtained with the 18S primers indicating isolates 13, 14 and 15 to be eukaryotic. Cluster tree analysis of the sequences using the contaminated sequences of isolates 13, 14 and 15 indicates that the contaminants were not closely related suggesting very different sources (Fig. 3.11). It is possible that these contaminants exist very closely in the environment with the eukaryotic species

While a number of researchers have found fatty acid analysis useful for bacterial identification (Logar *et al.*, 2001) the results in this study were disappointing. Analysis of all isolates was inconclusive at best, and gave no level of identification at worst. It was of some use as an indicator when the sequencing results were undecided, for example, isolate 9 was identified by sequencing as an *Enterobacter* spp. and by FAME analysis as similar to isolate 7, which was identified by sequencing as *P. agglomerans.* This suggests that isolate 9 may be *P. agglomerans* or closely related. The cluster diagram from the FAME analysis provided little useful information (Fig. 3.8). Daane *et al.,* (2001) who isolated and characterised 51 bacteria associated with the rhizosphere of salt marsh plants found similarity matches were often much lower for FAME analysis than sequencing.

FAME analysis was not useful for identifying isolates 13 and 14 as yeasts or isolate 15 as a fungus, possibly attributable to contamination. In line with this, an evaluation of FAME analysis for the identification of clinically isolates yeasts

concluded that this approach, with the current database, was not suitable for the routine identification of clinically important yeasts (Christ Jr *et al.,* 1996).

Sequencing of the 16S rRNA gene is often used as a method of identifying bacteria as it is considered to have minimal variation. However, direct sequencing using the 27f primer region proved problematic for many of the isolates and cloning and sequencing of two of the isolates revealed gene sequence microheterogeneity within the 16S rRNA subunit. Microheterogeneity of rRNA gene clones, in this case, could be due to allelic variation within or between members of a population, polymerase incorporation errors and/or sequencing errors. The rate of the *Taq* polymerase enzyme incorporation errors may vary 5-fold (~2 x 10^{-4} to <1 x 10^{-5} per nucleotide per cycle) according to the precise DNA sequence and the *in vitro* condition of DNA synthesis (Hansen *et al.,* 2001), while the sequencing process has a greater than 98.5% accuracy at 700base pairs (personnel communication, Thorn, 2003). That it was problematic to sequence the same section (the 27f primer region) of many of the isolates indicates that the microheterogeneity would be largely due to allelic variation in that region. The concentration of base variations in specific areas, as demonstrated by the clones (Fig. 3.12 and Fig. 3.13), also suggest a region of microheterogeneity rather than inherent protocol error.

The three clones sequenced for isolate 15 and the three for isolate 6 were distinct. BLAST results from all six clones indicated *Enterobacter agglomerans* as the closest match. Alignment of the clone sequences with an *Enterobacter agglomerans* full length 16S rRNA gene sequence obtained from the NCBI database allowed determination of the base positions at which variability occurred among the clones of the same isolate. Stackebrandt and Goebel (1994) claim that if the sequence homology of the 16S rDNA is less than 97% between two organisms it is unlikely they will exhibit more than 60 – 70 % DNA similarity and hence that they are related at the species level. It is therefore possible that isolate 6 is not *Enterobacter agglomerans,* however alignment with this sequence still illustrates the regions of variability between the clones. For this reason regions are counted as variable only when differences occur between the clone sequences at the same base pair position and not between the clones and the complete 16S *Enterobacter agglomerans* sequence.

Fig. 3.12 shows a section of the alignment of the clones obtained from isolate 15 indicating variability between base positions 398 and 420.

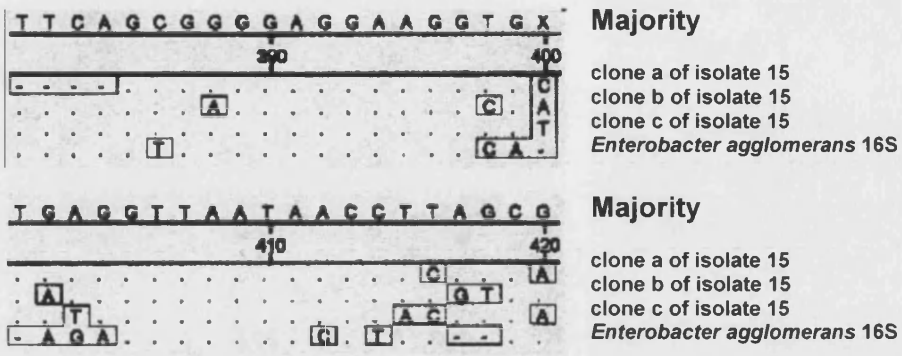

FIG. 3.12 Alignment of isolate 15 clones with full length *Enterobacter agglomerans* 16S rRNA gene sequence. This is a region of hypervariability, with variation in the same base positions between all 3 clones (398bp – 420bp).

(·) bases that match the consensus exactly

(□) bases that differ from the consensus

(-) no base

Clones of isolate 6 exhibited regions of variability at positions 233 to 237, from 508 until 527, from 1133 to 1138 and 1167 to 1172. The last two regions are shown in Fig. 3.13.

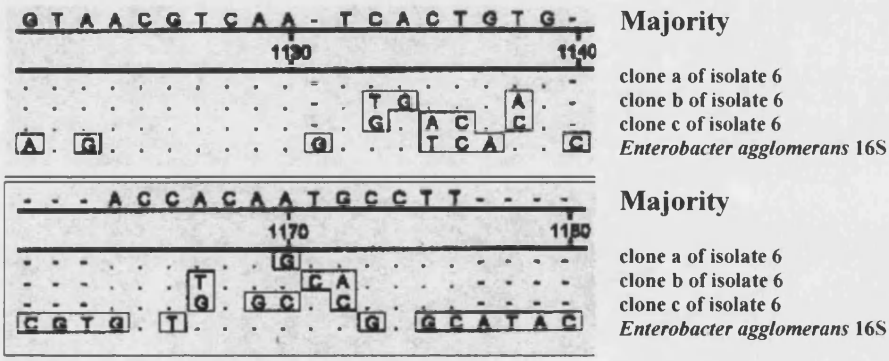

FIG. 3.13 Alignment of isolate 6 clones with full length *Enterobacter agglomerans* 16S rRNA gene sequence, showing variability between the clones in base pair positions 1133 to 1138 and 1167 to 1172.

(·) bases that match the consensus exactly

(□) bases that differ from the consensus

(-) no base

That these levels of variation were experienced with 2 out of 15 isolates sequenced indicates that variation within the 16S rRNA gene may be more common than is generally accepted. The regions of variability, however, appear to vary among species. Although more sequencing of both strands would be required to prove this.

The difficulties experienced in this investigation while trying to sequence the 16S gene is in agreement with Pascual *et al.*, (1995) who found it problematic to sequence this region of the genome of *Corynebacterium minutissimum* and attributed the difficulty to sequence microheterogeneity in the 16S rRNA region.

Ueda *et al.*, (1999) studied the frequency of heterogeneity among the multiple 16S rRNA genes within a single microorganism (*Streptomyces* strains). They sequenced a 120bp region from 158bp to 278bp and determined the existence of 136 heterogeneous loci among a total of 33 strains. The heterogeneous loci were detected only in positions 174 to 182 and 187 to 196 within the hypervariable α region which possibly forms a stem and loop structure and is designated helix 10.

In addition to this is the problem derived from the organisation of rRNA genes (rDNA) as a multigene family, often as *rrn* operons in bacteria. The differences that exist between the different operons are ignored when sequences are obtained after cloning of a single operon or directly from polymerase chain reaction products. Direct sequencing of PCR products, such as in this investigation, produces a mean sequence in which mutations present in the most variable domains are hidden. Cloning a single operon results in a sequence that differs from that of the other operons and of the mean sequence by several point mutations (Cilia *et al.,* 1996). It would therefore be optimal to sequence or clone a number of PCR products to ensure correct identification at the species level or below. This would, however, substantially increase the length of time and cost of the investigation, both of which may be well spent for a phylogenetic study but less so for the purposes of this investigation.

Problems were also encountered in attempts to use the I-341f and I-354r primers. These primers were designed by Watanabe *et al.*, (2001) after comparison of sequence data from newly recognised groups with a variety of universal bacterial primers revealed a number of mismatches. Inosine residues were introduced at

positions where mismatches were frequently found. Their investigation revealed that denaturing gradient gel electrophoresis fingerprint of bacterial groundwater populations detected a phylotype and two sequences not detected by the two other universal 16S rRNA bacterial primers. They concluded that the introduction of inosines into degenerate nucleotide positions is useful for detecting more diverse populations in the environment and reducing biases that may occur during PCR amplification. Only very small amounts of product could be obtained using this primer pair. It was concluded that these primers while useful for amplifying 16S fragments from environmental heterogeneous DNA, they were of less use for identification (personnel communication K. Watanabe, 2001).

Sequencing of the internally transcribed spacer region between the 16S and 23S rRNA genes is also commonly used for bacterial identification. This, however, has the disadvantage of including several tRNA genes and noncoding regions which appear to be under less selection pressure and are therefore more variable than the 16S and 23S genes themselves (Louws *et al.*, 1999). Sequencing of the 23S rRNA gene has also been used for identification and although the overall phylogenetic information content of this sequence is greater than that of the 16S, the number of currently available complete 23S sequences is much smaller than for the 16S rRNA gene (Ludwig and Schleifer, 1999).

Information obtained from the sequence of the 16S gene still represents the most useful, accessible and accurate method of bacterial identification. The results, should however, be interpreted with the limitations in mind (Stackbrandt and Goebel, 1994). This is particularly relevant to interpretation and differentiation of species within the *Enterobacteriaceae* (Hedegaard *et al.*, 1999). A number of isolates from this investigation were identified as belonging to this group by both 16S sequencing and FAME analysis. Some studies have suggested that this group may be polyphyletic (Mollet *et al.,* 1997), meaning a taxon that shares derived characteristics which originated several times by convergence. These taxa are generally considered invalid or unnatural groupings (Palaeos, 2003). The essential problem caused by this grouping is that assumptions are made that species are related, when in fact they are derived from different ancestors (Bunyard and Velez, 2001).

Comparison of the levels of antagonism exhibited and enzymatic production by isolates did not reveal any immediate correlations between the two, suggesting

that other modes of action are either responsible for, or are certainly contributing to the antagonism. However, two of the *Enterobacter agglomerans* strains and *Buttiauxella noackiae* displayed strong cellulolytic activity. Cellulase (β-1,4-glucanase) significantly retards the development of zoosporangia and chlamydospores of *Phytophthora cinnamomi* (Downer *et al.*, 2001a). Further analysis including *in vitro* testing for inhibition of *P. infestans* by the purified enzyme is required.

The phenotypic tests agree with the sequence identification predominantly, although there are exceptions. Isolate 1, identified as a strain of *P. putida,* is oxidase negative, however all Pseudomonads are positive in cytochrome *c* oxidase except *P. maltophilia* and phytopathogens. The former species produces a yellow pigment on nutrient agar excluding isolate 1 and the latter by definition also excludes isolate 1. Biochemical reactions of bacteria are often heavily dependent on the conditions in which they are cultured, for example medium and temperature (Stolp and Gadkari, 1981) which may be influencing the response of isolate 1.

Isolate 1 had blue fluorescence under UV light on KB medium (Fig. 3.2). Blue-green fluorescence is considered indicative of *Pseudomonas aeruginosa* although this isolate was identified as having 98% similarity to *P. putida* based on 16S sequence information. *P. aeruginosa* is best known as an opportunistic pathogen although rhizobacterium strains have been used as BCAs (Bano and Musarrat, 2003). The sequence cluster diagram (Fig. 3.10) indicates that isolate 1 and 2 (identified *P. putida* 99% by sequencing) are more closely related to each other than to isolate 3 (also identified *P. putida* 99% by sequencing). Pyocyanin is responsible for the blue-green colour associated with *P. aeruginosa* (Lemoual, 2002). The blue fluorescence by isolate 1 was observed on KB which enhances fluorescein but inhibits pyocyanin production (Sigma, 2003). This combined with the sequence results suggests that isolate 1 is more closely related to *P. putida* but produces at least one different pigment.

There has been a large amount of work published involving the use of various strains of *Pseudomonas putida* as a BCA against a wide range of plant diseases including a number caused by oomycete pathogens such as *Pythium* spp. (Harris and Adkins, 1999). The mode of action of certain strains has been studied extensively and has been demonstrated to reside mostly in the production of

antibiotics and iron-chelating siderophores. There are slight base variations among the three *P. putida* (*Arthrobacter siderocapsulatus*) strains in this study and published strains. The phenotypic characterisation profiles of these three strains also differed, which suggests that the minor base differences are significant. The modes of action by which these strains antagonise *P. infestans* may also be different. Chin-A-Woeng et al., (2003) recently published a list of reasons why *Pseudomonas* spp. are particularly suitable as BCAs. These are that they i) can use many exudate compounds as nutrients, ii) are abundantly present in natural soils, in particular on plant root systems, which is indicative for their adaptive potential, iii) have a high growth rate relative to many other rhizosphere bacteria, iv) possess diverse mechanisms by which they can exert inhibitory activity towards phytopathogens and thereby mediate crop protection, including the production of a wide range of antagonistic metabolites, v) are easy to grow *in vitro,* vi) can subsequently be reintroduced into the rhizosphere by seed bacterisation and vii) are susceptible to mutation and modification using state of the art genetic tools. *P. putida* has also been shown to have potato plant growth promoting properties, mediated by siderophore production (Bakker *et al.,* 1987), and to be an efficient root coloniser (Espinosa-Urgel, 2002).

Bacillus spp. are also frequently cited in the literature as effective BCAs. These Gram-positive bacteria have the application advantage of high thermal tolerance, rapid growth in liquid culture, ready formulation of resistant spores, and are considered to be safe as BCAs (Shoda, 2000). A strain of *B. thuringiensis* was selected by Sadfi *et al.,* (2002) as effective against the causal agent of Fusarium dry rot of potatoes. The *B. thuringiensis* strain from this investigation (isolates 10) matched 100% with a strain that has been employed in a phylogenetic study of *B. cereus* and *B. thuringiensis* in Norwegian soils (Ticknor *et al.,* 2001). This study used fluorescent amplified fragment length polymorphism analysis and sequencing of the 16S rRNA gene, results from both methods were consistent with each other but the former method provided a much higher resolution (Ticknor *et al.,* 2001). This suggests the possibility that the strain from the Norwegian soil and the strain from this investigation, while being very similar may not be identical.

Pantoea (formerly *Enterobacter*) *agglomerans* (isolates 5, 6, 7, 8 and possibly 9), although not as commonly studied as the above two genera in the context of biocontrol, has shown potential as a BCA effective against a range of postharvest

diseases on pome fruits (Nunes *et al.,* 2002). In this case the bacterium was shown to be effective under a wide range of temperatures and atmosphere conditions, which are useful characteristics for a BCA. In addition this species has been investigated from the standpoint of formulation. Preliminary work has determined agents and media which maximise bacterial viability when freeze-dried (Costa *et al.,* 2000) and spray-dried (Costa *et al.,* 2002).

The yeast genus, *Trichosporon*, has mostly been studied in the context of postharvest disease. For example, *Trichosporon* species have been demonstrated to control grey mold (*Botrytis cinerea*) and blue mold (*Penicillium expansum*) of apple fruits ($p < 0.05$, Tian *et al.,* 2002). In addition an unculturable *Trichosporon* spp. has shown potential against root rot of avocado, caused by *Phytophthora cinnamomi* (Menge, 2000).

Isolate 15 was identified as the fungus *Galactomyces geotrichum*, the anamorph of this being *Geotrichum candidum.* Species of the genus *Geotrichum* have been most commonly associated with causing plant disease, for example causing sour rot of citrus fruits (Droby *et al.,* 1998). An avirulent form of *Geotrichum candidum* has, however, been effective against postharvest rots on various fruit (Skaria, 2003).

Enterobacter amnigenus (isolate 4), *Citrobacter gillenii* (isolate 11, previously *C. genomospecies*) and *Buttiauxella noackiae* (isolate 12) have not been documented to my knowledge as BCAs.

CHAPTER IV

Mode of Action

4.1 INTRODUCTION

To determine and understand the mechanisms by which potential BCAs antagonise pathogens is extremely important for producing a successful biocontrol strategy (Guetsky *et al.,* 2002; Siddiqui and Shaukat, 2002). For example, a combination of BCAs which attack at different stages of the pathogens life cycle, will improve efficacy and reduce variability of disease control. Conversely, a BCA that inhibits the disease predominantly by the production of siderophores will not be very effective if applied in an area where iron is not limiting. In addition, application of a consortium of BCAs which operate by different modes of action will avoid placing a single selection pressure on the pathogen population and therefore decrease the likelihood of resistant races developing. This could be particularly important given the demonstrated ability of the *P. infestans* population to evolve, thereby circumventing control measures.

One major purpose of using an *in vivo* selection method was to ensure that potential BCAs operating by either direct or indirect modes of action were targeted. Direct methods include inhibition of the pathogen by the production of antimicrobial compounds (antibiosis), competition for iron through the production of siderophores, competition for colonisation sites and nutrients and parasitism that may involve secretion of extracellular cell wall degrading enzymes (Whipps, 2001).

Antibiotics consist of a chemically heterogeneous group of organic, low-molecular weight compounds produced by microorganisms. A large variety of studies has shown that bacterial biocontrol strains exhibit a wide range of diversity in type and number of antibiotics produced (Raaijmakers *et al.*, 2002). Inhibition of *P. infestans* growth on Petri dishes by culture filtrates or compounds diffusing from antagonist colonies would suggest the production of antibiotics by the bacterial antagonist.

Iron, while abundant in the earth's crust, is predominantly found in the highly insoluble form of ferric hydroxide and is therefore only available to organisms at concentrations at or below 10^{-18} M in soil solutions at neutral pH. Bacteria require iron at micromolar concentrations for growth and so have developed the ability to produce siderophores (Handelsman and Stabb, 1996). These compounds are low molecular weight, ferric-specific iron-chelators that are transferred into the microbial cell by specific membrane receptors (Jacques *et al.*, 1995). Their biosynthesis is regulated by the availability of iron in the surrounding medium and, under conditions of high iron concentrations, the production of these compounds is repressed (Machuca and Milagres, 2003). Siderophore production and uptake of the iron-siderophore complex provide the organism with a competitive advantage under conditions of low iron. This may contribute directly or indirectly to preventing establishment of a pathogen in the rhizosphere (Dowling and O'Gara, 1994).

Chemically, most siderophores can be classified as either hydroxamates or catechols depending on the binding site of the Fe^{3+} (Neilands, 1993) and despite the considerable structural variation all siderophores form six-coordinate octahedral complexes with the ferric ion (Guerinot, 1994). The fluorescence produced by *Pseudomonas putida* isolates 1, 2 and 3 (observed in chapter III) is characteristic of fluorescent compounds called pyoverdines or pseudobactins which function as siderophores in this group of microorganisms (Paulitz and Loper, 1991). Pseudomonads also produce pyochelin, a siderophore with a slightly lower affinity for iron, and its precursor salicylic acid (Whipps, 2001), the latter also being able to function as an iron chelator (Guerinot, 1994). The role of salicylic acid as a plant signal molecule makes this particularly interesting. Siderophores are also known to induce systemic acquired resistance to plant pathogens (van Loon *et al.*, 1998).

Siderophore production is commonly determined by variations of the universal assay of Schwyn and Neilands (1987). This assay is based on competition for iron between the ferric complex of an indicator dye, chrome azurol S (CAS), and a chelator or siderophore produced by microorganisms. The siderophore has a higher affinity for iron (III) and therefore removes it from the CAS complex causing a colour change from blue to a range of colours in the orange, red and purple spectrum (Milagres *et al.*, 1999).

Lytic enzymes may include ß-1,3-glucanases, proteases, lipases and cellulases. The cell wall of *P. infestans* is 80 - 90% ß-1,3-glucans and so microorganisms producing enzymes that degrade these compounds may be effective at inhibiting the pathogen and therefore the disease it causes (McLeod *et al.,* 2003). Likewise cellulose (ß-1,4-glucan) is a component of oomycete cell walls and enzymes that degrade it may be effective against the pathogen (Downer *et al.,* 2001[b]). The characterisation tests (**3.3.1**) have shown cellulolytic activity by isolates 3, 14 and 15. Chitinases have been shown to play a role in biocontrol antagonism, for example in the suppression of *Botrytis cinerea* by *Serratia plymuthica* (Frankowski *et al.,* 2001). Oomycetes, however, contain no chitin in their cell walls and these enzymes are therefore unlikely to contribute to the mode of action and their activity was not measured.

A well documented indirect mechanism of disease inhibition is by activation of the plant defences. Root colonisation by rhizobacteria can induce systemic resistance (ISR) in plants to pathogen infection (Persello-Cartieaux *et al.,* 2003). In order to study ISR it is necessary to develop a gnotobiotic system in which the foliage is prevented from having contact with the test antagonists which are used to inoculate the roots. Therefore any resistance to disease observed in the foliage can be attributed to induced resistance.

Other possible modes of action include inactivation of pathogen germination factors present in seed and root exudates and degradation of pathogenicity factors of the pathogen such as toxins. None of these mechanisms is necessarily mutually exclusive and it is common for one organism to exhibit multiple modes of action. For some BCAs, different mechanisms or combinations of mechanisms may be involved in the suppression of different plant diseases (Whipps, 2001).

4.2 MATERIALS AND METHODS

See appendix IV for media constituents

4.2.1 Antibiosis

4.2.1.1 *In vitro* antagonism between *P. infestans* and potential BCAs

In vitro antagonism was studied on Petri dishes containing 15ml 30% V8 agar and 30% V8 agar supplemented with a filter sterilised solution of $FeCl_3$ to give final concentrations of 0.25g L^{-1} (V8.25) and 0.5g L^{-1} (V8.5). A plug of *P. infestans* (cork borer size 4) was inoculated in the centre of the plate and a loopful of antagonist at opposite edges of the Petri dish (Fig. 4.1). Plates were inverted and incubated at 15°C and growth of *P. infestans* was measured along axes C and D (Fig. 4.1) at 9 and 14 days. Strains of *Pantoea agglomerans* (isolates 5, 6, 7, 8 and 9) exhibiting suppression were retested for antagonism on V8 plates containing arginine, histidine and / or leucine. The amino acids were added in a quantity that provided nitrogen at 0.06 g L^{-1} (Wodzinski and Paulin, 1994). This was to test if certain amino acids reversed antagonism of *P. infestans.* Individual or combinations of amino acids have been shown to reverse antagonism of *Erwinia amylovora* by antibiotics from *P. agglomerans* (Wright *et al.*, 2001).

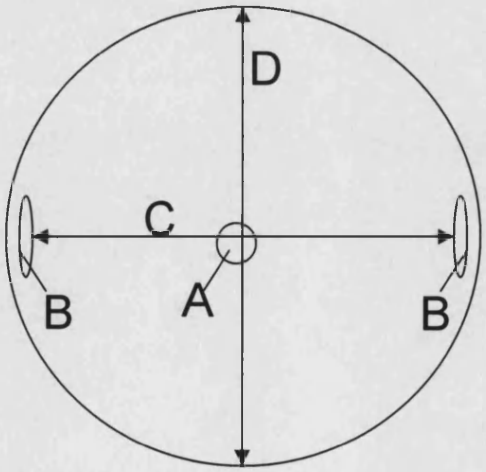

FIG. 4.1 Antibiosis plate. A = plug of *P. infestans,* B = antagonists, C and D = axes along which *P. infestans* growth measured.

4.2.1.2 Antibiosis by culture filtrates of potential BCAs

Isolates were grown in minimal liquid media (MLM) (Eckwall and Schottel, 1997) and incubated at 20 to 25°C on a shaker (150 rpm). Growth curves were determined by measuring absorbance at 650nm. Samples were taken immediately after inoculation, during the exponential growth phase, the onset of the stationary phase and a length of time after the culture had reached stationary phase as determined by the growth curve. The samples were centrifuged (2500 *g* for 10 min), filtered (0.22µm membrane) and 5ml was combined with 15ml 30% V8 agar at 45°C and poured into a Petri dish (Minkwitz and Berg, 2001). After the plates had set they were inoculated with a plug (cork borer size 4) of *P. infestans.* The plates were incubated at 15°C and growth was measured after 14 days.

4.2.2 Interaction between the antagonists and *P. infestans*

Petri plates of thin (5ml) 5% V8 were inoculated as for the antibiosis plates (Fig. 4.1). This was in order to study any visible effects of diffusible substances produced by the antagonist on the pathogen hyphae. Plates were also prepared with the antagonists inoculated 1cm either side of the plug of *P. infestans* to study possible hyphal colonisation. An inverted microscope was used to examine these interactions.

4.2.3 Hyphal colonisation

Hyphal colonisation was measured using a hyphal column assay (Yang *et al.,* 1994). An agar disc containing hyphae of *P. infestans* was dropped into a 250ml flask containing 100ml CD-V8 liquid medium. The flask was incubated by shaking it slowly at room temperature for 2 days. Then the preparation containing the agar disc was blended with a homogenizer and incubation was continued for another 5 days. The hyphal mass was washed with sterile water to remove the CD-V8 broth. A 4g portion of the washed hyphal mass was placed in a 5ml polypropylene syringe which was plugged with glass wool. This amount of hyphae filled the syringe to a volume of about 4.5ml. The hyphal column was then washed with 20ml of 0.01M phosphate buffer (pH 7.0, used throughout this experiment) before the bacteria were added.

Bacteria were grown for about 15 hrs in TSB (Oxoid). The cells were centrifuged, washed with phosphate buffer, and resuspended in phosphate buffer. A 2ml sample of the bacterial suspension prepared as in section **2.2.5,** was added slowly to the hyphal column. The actual number of total bacteria added to the column was quantified in each experiment by dilution plating. After 5 min of equilibration the hyphal column was washed with 2ml of phosphate buffer. Three additional washes, each with 2ml of phosphate buffer, were performed to remove most of the free bacteria. The number of cells removed from the column after each wash was determined by dilution plate counting and was subtracted from the total number applied to the column to calculate the percentage of cells retained in the hyphal column.

4.2.4 Siderophore production

A number of approaches were used to measure siderophore production.

4.2.4.1 Siderophore production in liquid medium (Scher and Baker, 1982)

Antagonists were grown in low iron (3.6µM $FeSO_4 \cdot 7H_2O$, to stimulate siderophore production) and high iron (0.5g $FeCl_3$ L^{-1}, to inhibit siderophore production) minimal liquid medium (Eckwall and Schottel, 1997) with the carbon source replaced with *P. infestans* cell walls (1% w/v). After incubation on a shaker (150 rpm, 5cm throw) for 5 days samples were centrifuged (2500 *g* for 10 min) and filtered (0.22µm membrane) and the pH of the filtrates adjusted to 5.5 with 1M NaOH. Each was added to two spectrophotometer tubes, 15µl of 10^{-2} M $FeCl_3$ was added to one tube, with the other tube being blank. Absorbance at wavelengths between 350 and 500 nm were measured against the blank (Scher and Baker, 1982).

4.2.4.2 CAS agar

Antagonists were inoculated on CAS agar (Schwyn and Neilands, 1987, Payne, 1994). Colour change was recorded.

4.2.4.3 Modified CAS agar

Antagonists were inoculated on modified CAS agar (Alexander and Zuberer, 1991). In order to provide a quantitative measurement, maximum width of the colour change was recorded.

4.2.4.4 Siderophore production in liquid medium (Alexander and Zuberer, 1991)

Antagonists were grown in flasks of modified M9 liquid medium (appendix IV), incubated at room temperature on a shaker (150 rpm, 5cm throw) until cultures had reached the stationary phase (determined by absorbance at 620nm). Samples were then centrifuged (2500 *g* for 10 min) and filtered (0.22µm membrane). Culture filtrates (1ml) were added to 1ml of modified CAS assay solution and allowed to equilibrate for 3 hours exactly before absorbance was measured at 630nm. When testing the siderophore concentration of isolate 12, samples (1ml) were also tested with 1.5ml modified CAS assay solution. M9 liquid medium was used as the blank and to zero the spectrophotometer. Deferoxamine mesylate, an iron specific chelator obtained from *Streptomyces* spp., was used to determine a standard curve for siderophore determination. In addition the absorption spectrum between 240nm and 470nm of the culture filtrate was measured i.e. the absorption spectrum of the ferric-siderophores complex and the colour noted.

4.2.5 Indoleacetic acid (IAA) production

IAA production was measured by the method of Bric *et al.,* (1991). NA was inoculated with the bacterial antagonists in grid patterns. Autoclaved dialysis tubing was laid on the plate which was then sealed and incubated at room temperature for 3 days. Whatman filter paper no. 2 was soaked in 2% 0.5M $FeCl_3$ in 35% perchloric acid (Salkowski's reagent). The dialysis membrane was then removed and placed on the filter paper, a red halo surrounding bacterial colonies in the membrane indicated IAA production. Additionally growth of *P. infestans* was measured on V8 agar plates containing 20µM indole-3-acetic acid.

4.2.6 ß-1,3-glucanase activity

Bacterial isolates were grown in minimal liquid medium (Eckwall and Schottel, 1997) with *P. infestans* cell walls as the sole source of carbon and inducer of β-1,3-glucanase activity. Flasks were maintained at room temperature and shaken at 150 rpm (5cm throw). Samples were taken at 0hr, 48hr, 96hr and 168hr and centrifuged (2500 *g* for 10 min) then filtered (0.22µm membrane). Culture filtrates were dialysed against 50mM sodium acetate buffer (pH 5.2) for 24hr at 4°C, then lyophilised and resuspended in 2ml 50mM acetate buffer, pH 5.2.

4.2.6.1 Alkaline copper method

ß-1,3-glucanase activity was measured by determination of reducing sugars using laminarin as the substrate (Dygert *et al.*, 1965).

The reaction mixture consisted of;

0.1 M sodium acetate buffer (pH 5.2)

1mg ml^{-1} laminarin

The laminarin substrate was dissolved in the acetate buffer by heating at 60°C before use

A 1ml enzyme extract was incubated with the reaction mixture (1ml) at 37°C for 1.5hr after which reagents A and B (Dygerts *et al.*, 1965) were added in equal volumes (see Table 4.1) to the reaction mixture, which was placed in a boiling water bath for 8 minutes and then cooled in running water. Controls consisted of the substrate with no enzyme extract, culture filtrate of growth medium with no bacteria and substrate with one unit of laminarinase. The absorbance was measured at 450nm using water as the blank.

TABLE 4.1 Recommended reagent volumes for various levels of reducing sugar (Dygert *et al.*, 1965).

Range	µg glucose	ml reagent A	ml reagent b
1	5 – 25	1	1
2	26 – 50	2	2
3	51 – 75	3	3
4	76 – 100	4	4
5	101 – 125	5	5

4.2.6.2 Glucose oxidase method

The same substrate reaction mixture was used as above (laminarin dissolved in sodium acetate buffer). Samples (1ml) were incubated with substrate (1ml) for 1.5hr. Table 4.2 contains the constituents of the glucose determination reaction mixture which were mixed in a microtest plate well. The enzyme hexokinase (10µl) was added to start the reaction. An ultra microplate reader (EL_X808, Bio-tek instruments, Inc., Vermont, USA) was used to record absorbance at 340nm. A standard curve was prepared from glucose.

Table 4.2 Constituents of the reaction mixture to measure ß-1,3-glucanase activity.

Components	Volume / µl
HEPES buffer (pH 7.5)	200
NAD (40mM)	10
ATP (100mM)	10
Glucose-6-phosphate dehydrogenase	10
Extract or standard	70 / 10

4.2.7 Protease activity

Bacterial isolates were grown in protease inducing liquid medium (Elad and Kaput, 1999). Cultures were incubated at room temperature on a shaker (150 rpm, 5cm throw). Samples were taken at 0, 48, 96 and 168hr, centrifuged (2500 *g* for 10 min) and filtered (0.22µm membrane). Culture filtrates were dialysed against 50mM sodium phosphate buffer pH 7.5 for 24hr at 4°C and were then lyophilised and resuspended in 2ml of the same buffer. Only culture filtrates from isolates 1, 13 and 10 were measured, as characterisation tests had showed proteolytic activity by these isolates (**3.3.1**). Isolate 14 exhibited no protease activity on skimmed milk agar and was used as a negative control.

A chromogenic method was used to quantify protease production (Mischke, 1996). Azoalbumin was dissolved in MOPS buffer (pH 7) to a concentration of 10mg/ml. 1 ml of the substrate and 1ml sample were mixed and incubated at 37°C for 0, 20, 40, 80 and 160 minutes. Enzyme activity was terminated when

undigested protein was precipitated by addition of 4ml of 10% trichloroacetic acid. The mixture was centrifuged 5 min (15 600 *g*). A volume (200µl) was removed and neutralised with 200µl 0.5N NaOH. The released colour was quantified as absorbance at 440nm. The control was the culture filtrate from sterile protease inducing medium. A standard curve was determined using proteinase K.

Samples were also tested by inoculating (50µl) skimmed milk agar (Chapter III). Clear agar zones indicated proteolytic activity.

4.2.8 Effect of enzymes on sporangia of *P. infestans*

Culture filtrates were tested for inhibitory activity against sporangia. Culture filtrate (100µl) was added to an equal volume of sporangia suspension (adjusted to a concentration of 5 x 10^4/ml) in a microtiter plate and allowed to equilibrate for 4 h. After incubation, fluorescein diacetate (FDA, 50µl) was added to each well and incubated for 5 minutes. FDA (10mg/ml acetone) was stored at -20°C and diluted (1:49) in holding buffer (see appendix IV) before use. The plates were observed under an Olympus inverted microscope (Model IMT) equipped with epifluorescence optics. The sporangia fluorescing green were counted as live and those not fluorescing as dead.

Protease positive samples were also incubated with a proteinase inhibitor cocktail (40µl), which had broad specificity for the inhibition of serine, cysteine, aspartic and metallo proteases. Samples were tested as above, using isolate 14 as the negative control.

4.2.9 Induced systemic resistance (ISR)

Bacterial suspensions were adjusted to an optical density of 0.4 at 620nm in 10mM potassium phosphate buffer (pH 7.2). Yeast suspensions were adjusted to a corresponding concentration using a haemocytometer. Suspensions of fungal spores were adjusted to an approximate concentration of 5 x 10^6/ml also using a haemocytometer. Controls were phosphate buffer only. Potato (*Solanum tuberosum* L. cv. King Edward) supplied by the Scottish Agricultural Science Agency (Edinburgh, SASA) was kept as sterile plantlets on MS basal medium supplemented with 30g sucrose (BDH Laboratory supplies, Poole, England) and 8g phytogel, L^{-1}. The method of SASA and the Centro Internacional de la Papa (CIP) training manual for potato plantlet propagation were followed. Plantlets were multiplied as single-node explants (1cm long) grown in Magenta vessels with vents (Sigma, Gillingham, Dorset). Plantlets were grown in a controlled environmental chamber at 22°C with a 12.5h/11.5h day/night cycle. Sterile stem cuttings were used as the starting material after storage overnight on solidified Murashige and Skoog basal medium (MS, as described above).

A number of approaches for testing ISR were tried as follows:-

4.2.9.1 Adapted method from Simons *et al.,* (1996).

An adapted version of the gnotobiotic system designed by Simons *et al.,* (1996), for studying tomato rhizosphere colonisation by plant growth-promoting *Pseudomonas* bacteria, was used. Muslin gauze was secured around the base of a piece of glass tubing (15cm, 37mm inner diameter and 41mm outer diameter). Sharp sand (60g) moistened with MS solution (2ml) was placed in the glass tube and shaken to compact it. Indicator tape was wrapped around the glass tube approximately ¼ from the base so that it could be wedged into a 150ml sterilin container (Bibby Sterilin Limited, Tilling Drive, Stone, Staffordshire). This was autoclaved and in aseptic conditions placed in the sterilin container containing 5ml solidified MS. This was sealed by wrapping nescofilm around the indicator tape. The sand was inoculated with 5ml bacterial solution and subsequently covered with 10ml solidified MS medium. The open end of the tube was plugged with cotton and capped with aluminium foil to prevent dehydration (Fig. 4.2).

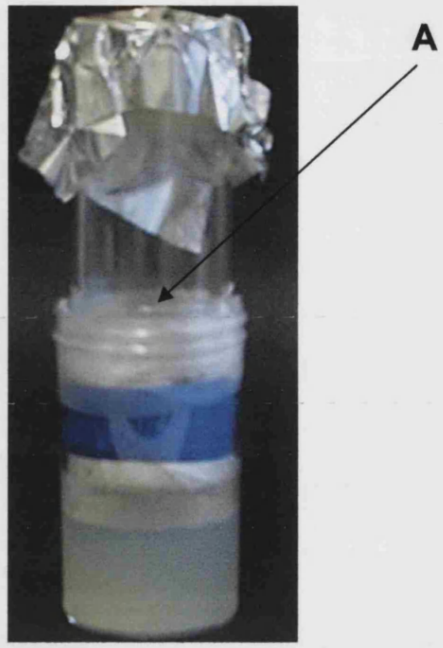

FIG. 4.2 Gnotobiotic system adapted from Simons *et al.*, (1996). A potato explant was placed in the MS medium in the glass cylinder (A). See section **4.2.9.1**.

4.2.9.2 Simons *et al.*, (1996) using Magenta vessels

A further adaptation of this method with Magenta vessels was tried. Magenta vessels with vented lids were obtained from Sigma. Sterile sand (60g) was placed in a sterile vessel and moistened with 2ml sterile MS liquid. This was inoculated with 10ml bacterial suspension and 15ml MS agar added. Potato explants were placed in the medium 48hrs later.

4.2.9.3 Magenta vessels using layers of medium

Murashige and Skoog (MS, recipe as in section **4.2.9**) medium (25ml) was poured into a Magenta vessel and allowed to cool. Antagonist suspension (10ml) was added to 10ml MS medium at 45°C, which was subsequently poured into the Magenta vessel. This was allowed to solidify and then a further 10ml of MS media was poured over this. Explants of potato cv. King Edward were then transferred to the vessels.

4.2.9.4 Magenta vessels without layer of medium

Non-autoclaved, microwaved (2min, medium power) MS medium (25ml) was poured into a sterile Magenta vessel which was then autoclaved. When the medium had cooled to 45°C antagonist suspension (5ml) was added and the

container gently shaken to mix. When the medium had solidified potato explants were transferred to the Magenta vessel. To ensure the foliage did not come into direct contact with the bacteria, leaves were taken from the plant at the end of the experiment and plated onto NA in order to examine bacterial growth. After three weeks the Magenta vessels were opened in aseptic conditions and sprayed with 5ml 5 x 10^4/ml suspension of *P. infestans* sporangia. The control was plantlets grown sterile MS medium, inoculated with sterile water. Disease lesions were counted after 2 weeks.

4.3 RESULTS

4.3.1 Antibiosis

4.3.1.1 *In vitro* antagonism between *P. infestans* and potential BCAs

In vitro antagonism between potential BCAs and *P. infestans* on 30% V8 agar (V8), 30% V8 agar supplemented with 0.25g $FeCl_3$ L^{-1} (V8.25) and 0.5g $FeCl_3$ L^{-1} (V8.5) is illustrated in Fig. 4.3, 4.4 and 4.5, respectively. A number of the isolates exhibited high levels of antagonism *in vitro*.

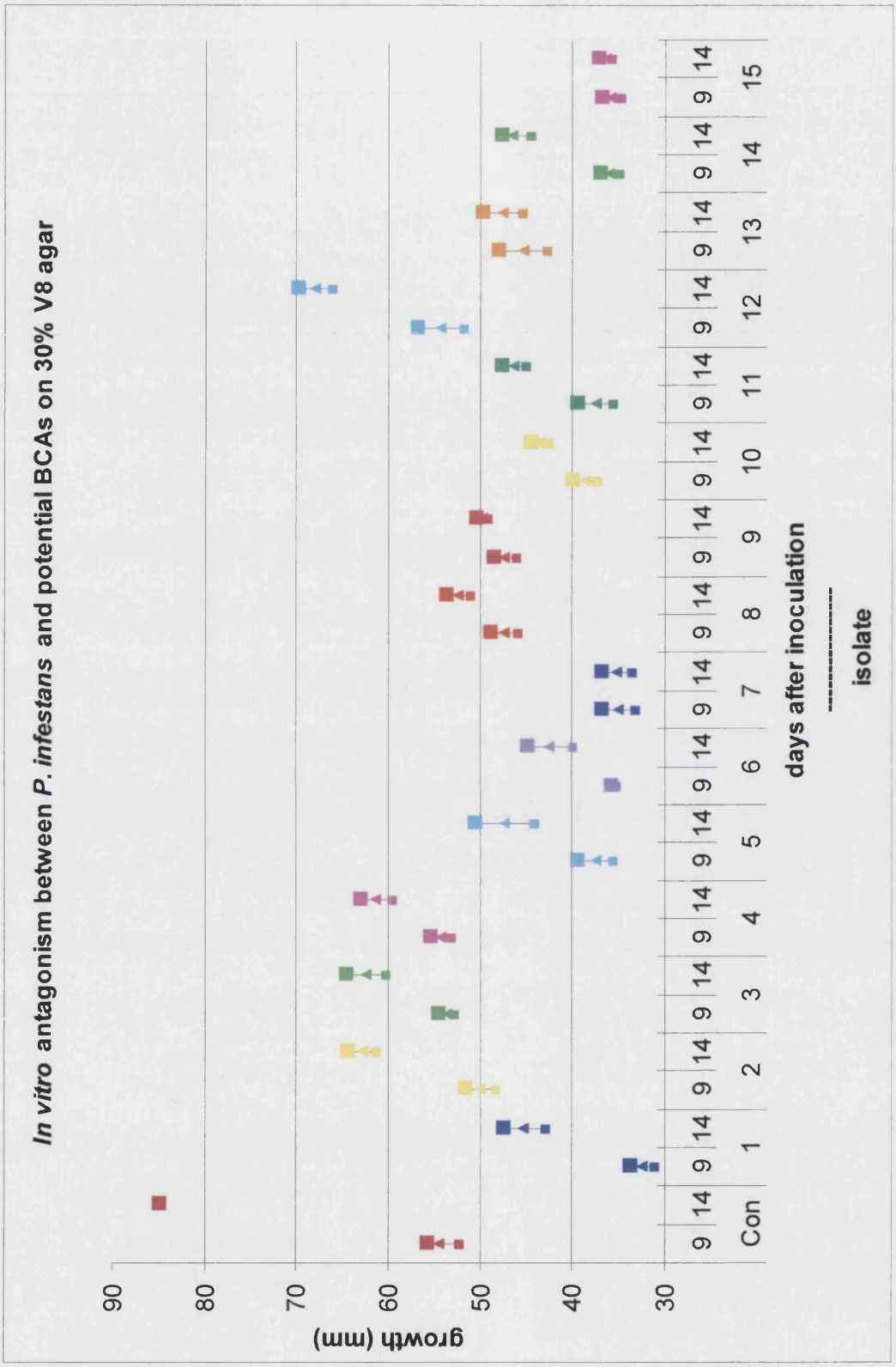

Fig. 4.3 Antagonism between potential BCAs and *P. infestans* on V8 agar. Points are means of six replications with standard error bars. Control (Con) was *P. infestans* grown on V8 agar.

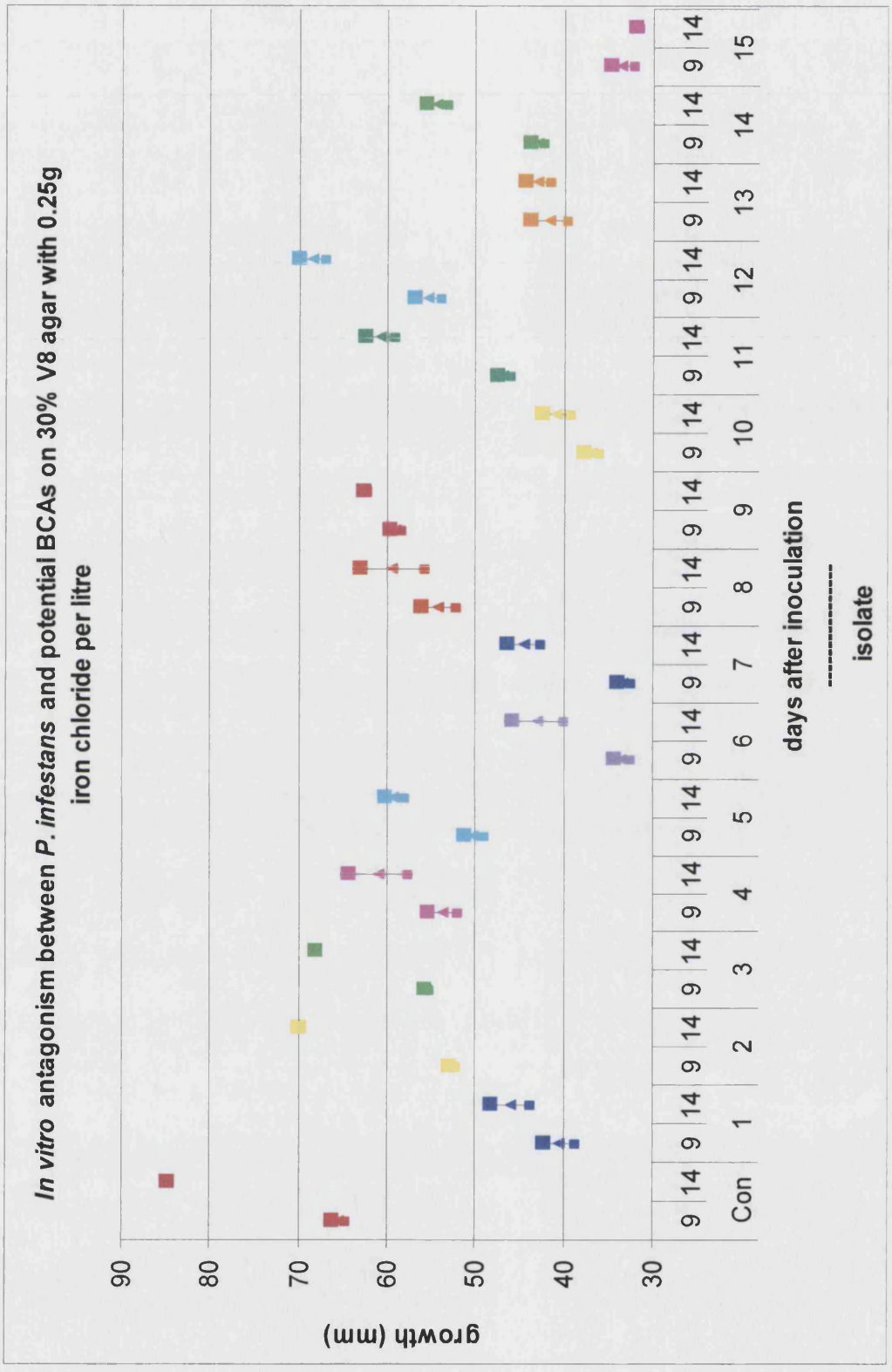

Fig. 4.4 Antagonism between potential BCAs and *P. infestans* on V8 agar supplemented with 0.25g per litre $FeCl_3$. Points are means of six replications with standard error bars. Control (Con) was *P. infestans* grown on V8 agar with 0.25 g L^{-1} $FeCl_3$.

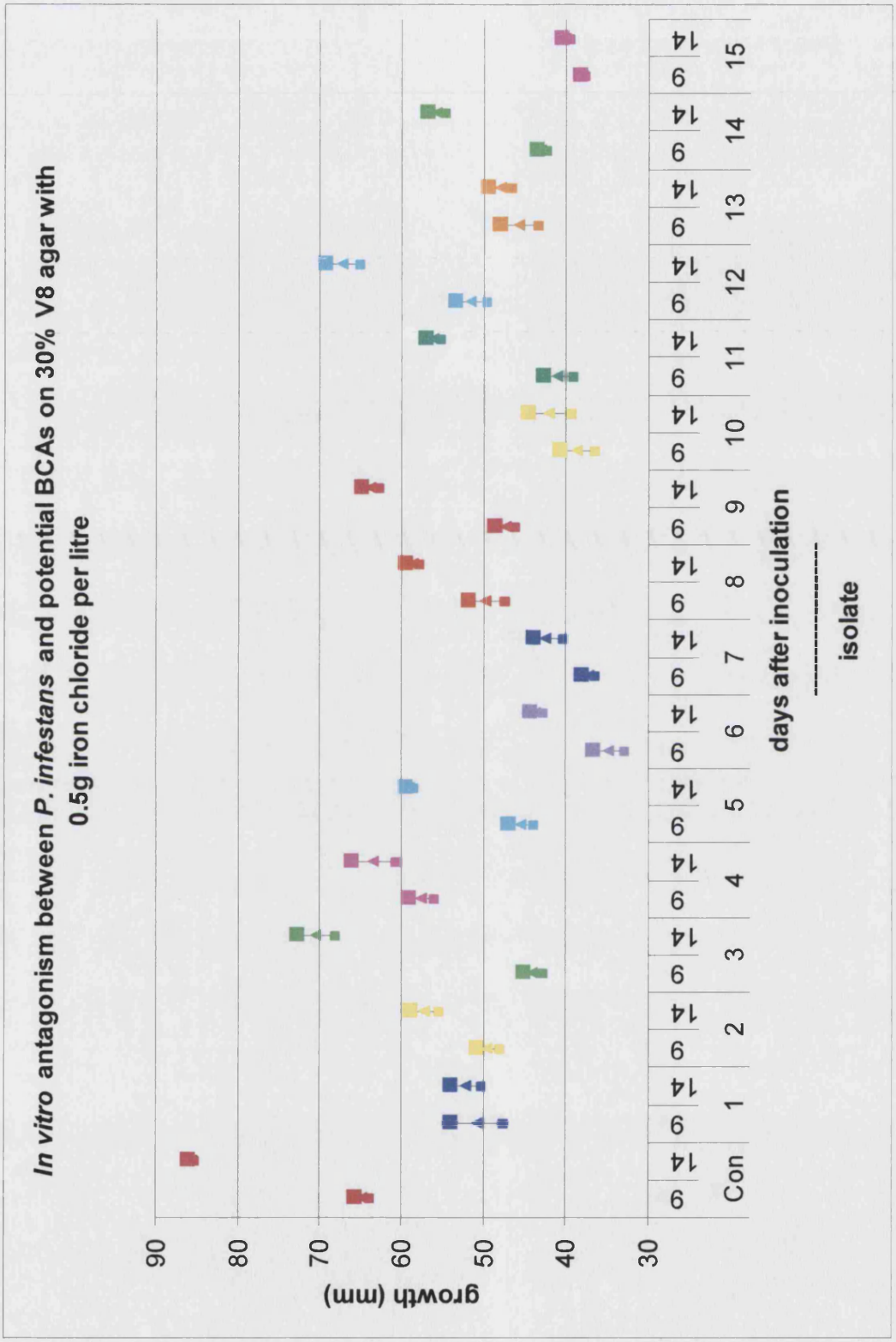

Fig. 4.5 Antagonism between potential BCAs and *P. infestans* on V8 agar supplemented with 0.5g per litre $FeCl_3$. Points are means of six replications with standard error bars. Control (Con) was *P. infestans* grown on V8 agar with 0.5 g L^{-1} $FeCl_3$.

Isolate 1, a *Pseudomonas putida* strain, significantly inhibited (p = 0.001) growth of *P. infestans* on all three media (Fig. 4.3, 4.4 and 4.5). The zone of inhibition measured at 9 d decreased by approximately 10mm on medium with each medium of increasing iron concentration. The final zone of inhibition measured at 14 d was the same on V8 and V8.25, but slightly lower on V8.5 (Fig. 4.6).

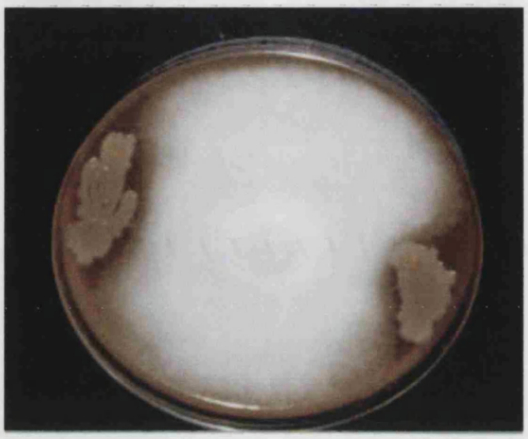

FIG. 4.6 Isolate 1 inhibiting *P. infestans* on high iron medium (V8.5), with a zone of inhibition evident after incubation for 14 d.

Isolates 2 and 3, both strains of *P. putida,* showed varied but consistently low levels of inhibition on all media (Fig. 4.3, 4.4, 4.5 and 4.7). The inhibition by isolate 3 on V8.5 was the lowest exhibited by all 15 isolates (Fig. 4.7).

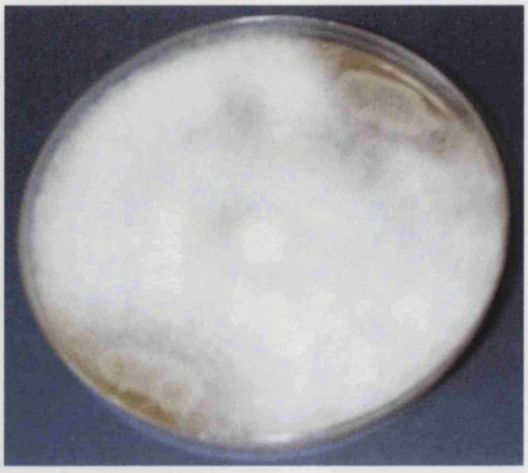

FIG. 4.7 Isolate 3 showing minimal inhibition of *P. infestans* on V8.5 after 14 d.

Isolate 4, *Enterobacter amnigenus*, and isolate 12, *Buttiauxella noackiae,* showed no evidence of inhibition by diffusible substances on any media. *P. infestans* did not, however, achieve the same level of growth as seen in the control as the mycelium did not completely cover the bacteria.

Isolate 5 displayed approximately 50% inhibition on V8 at 9 d (Fig. 4.3) decreasing on media with higher iron levels (Fig. 4.4 and 4.5). A zone of inhibition is evident by isolate 5 on V8 (Fig. 4.8) indicating the production of diffusible compounds. Inhibition was lower on media supplemented with iron, decreasing from approximately 50% to 70% at 14 d (Fig. 4.4 and 4.5).

FIG. 4.8 Isolate 5 antagonising *P. infestans* on V8 only with diffusion zones evident after 14 d.

Isolate 6 exhibited inhibition of *P. infestans* by approximately 50% on all three media (Fig. 4.3, 4.4 and 4.5) with zones of inhibition evident. Isolate 7 was extremely inhibitory towards *P. infestans* growth on V8, even after 14 d with inhibition on V8 supplemented with iron decreasing slightly by the 14th day. There were definite zones of inhibition indicating the production of diffusible substances (Fig. 4.9).

FIG. 4.9 Isolate 7 inhibiting *P. infestans* on V8 only at 14 d after inoculation

Isolate 8 and 9 inhibited *P. infestans* by 40% with some evidence of diffusible substances (Fig. 4.10). The level of inhibition decreased by approximately 10% on media supplemented with iron.

FIG. 4.10 Inhibition of *P. infestans* by isolate 8 on V8 only after 14 d.

All *Enterobacter* strains with similarities to *E. agglomerans* (isolates 5 to 9) showed some evidence of zones of inhibition. Incorporation of arginine, histidine and/or leucine into the media did not reduce inhibition of *P. infestans* by isolates 5, 6, 7, 8 and 9.

Isolate 10 exhibited inhibition of *P. infestans* by approximately 50% on all three media (Fig. 4.3, 4.4 and 4.5). Zones of inhibition were evident on two of the V8 plates for isolate 10; however, these zones were not evident on the rest of the plates (Fig 4.11).

FIG. 4.11 Inhibition by isolate 10 on V8 only (left) and V8.5 (right) after 14d.

Isolates 11 and 14 display similar patterns, approximately 50% inhibition occurred on V8 at 9 days (Fig. 4.3) decreasing on media with higher iron levels (Fig. 4.4 and 4.5).

Isolate 13 showed high levels of pathogen antagonism on all media although no zone of inhibition between the antagonist and *P. infestans* could be seen (Fig. 4.12). Isolate 15 antagonised in a similar manner (Fig. 4.13), inhibiting pathogen growth by over 50%. On V8 medium both antagonists inhibited growth along both axis's C and D (Fig. 4.1) unlike any of the other isolates. This is still evident on higher iron medium for isolate 15 but not isolate 13 (Fig. 4.12b and 4.13b).

FIG. 4.12a Inhibition by isolate 13 on V8 only medium after 14 d.

FIG. 4.13a Inhibition by isolate 15 on V8 only medium after 14 d.

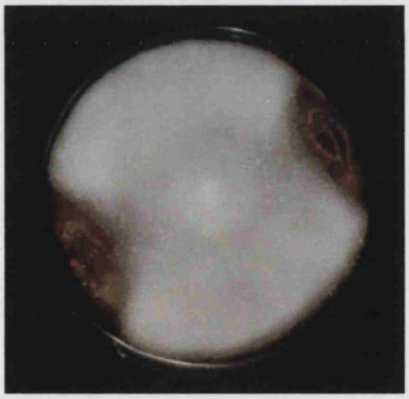

FIG. 4.12b Lower inhibition by isolate 13 on V8.5 medium after 14 d.

FIG. 4.13b Inhibition by isolate 15 still evident on high iron medium (V8.5) in all directions after 14 d.

4.3.1.2 Antibiosis by culture filtrates of potential BCAs

The growth curves were similar for most of the isolates. A very short lag phase, followed by a reasonably sharp exponential growth phase reaching stationary phase by approximately 22h (Fig. 4.14). All 15 isolates showed no indication of reaching declining (death phase) by 150h (Fig. 4.14, 4.15, 4.16 and 4.17). Samples of culture filtrates from isolates exhibiting the same growth pattern as Fig. 4.14 (isolates 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 13 and 15) were collected at 0, 14, 22 and 42h after inoculation in order to test each growth phase for antibiotic activity.

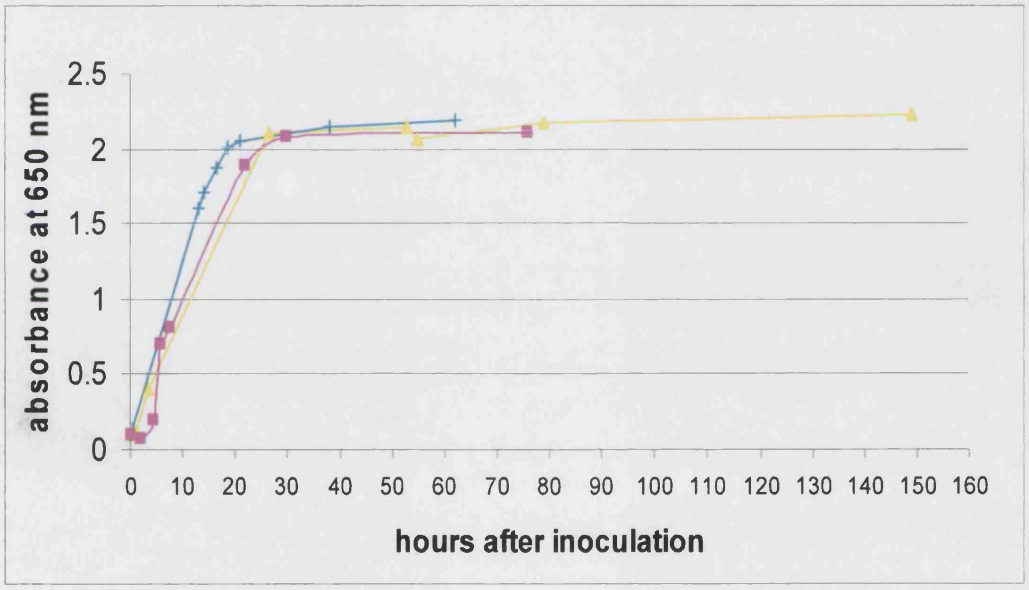

FIG. 4.14 Growth curve of isolate 13. The experiment was repeated seven times and from this data growth curves were constructed. Trends with anomalous results were not included. Samples were collected at 0h, 14h, 22h and 42h after inoculation.

Of the potential BCAs exhibiting this growth trend the culture filtrates of isolates 4, 5, 8 and 10 showed no antibiotic activity against mycelial growth of *P. infestans*. The culture filtrate of isolate 1 extracted at 14h inhibited *P. infestans* growth by approximately 14% increasing to 20% when extracted at 22h. By 42h the culture filtrate has almost completely lost inhibitory activity (Fig. 4.15).

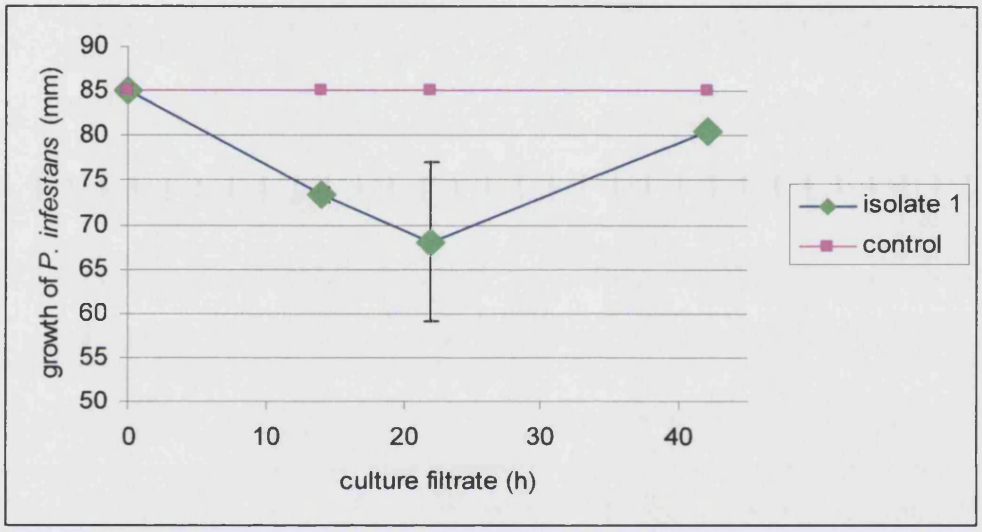

FIG. 4.15 Inhibitory activity of culture filtrates of isolate 1 extracted after 0h, 14h, 22h and 42h incubation. Culture filtrates (5ml) were combined with V8 agar (30%; 15ml) and inoculated with a plug of *P. infestans*. Mycelial growth was measured after 14 days. Points are means of three replications with standard error bars. The control was *P. infestans* grown on V8 (30%) agar only.

The culture filtrates of isolates 2 and 7 demonstrated similar inhibitory patterns to isolate 1. Antibiotic activity was greatest in the 22h extract, with the culture filtrate of isolate 7 suppressing growth of *P. infestans* by 25% and the culture filtrate of isolate 2 suppressing growth by 15%. Inhibitory activity decreased to 0 by 42h with both isolates (Fig. 4.16).

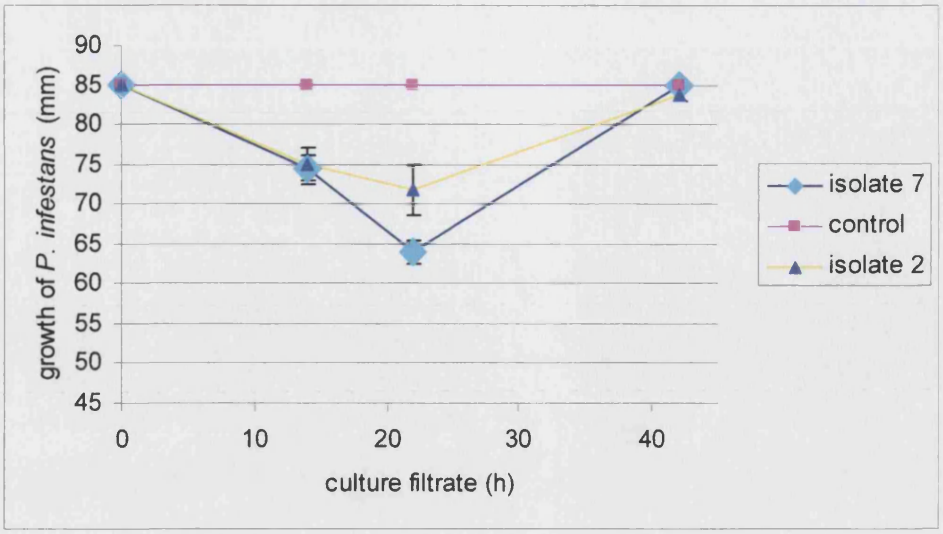

FIG. 4.16 Inhibitory activity of culture filtrates of isolates 2 and 7 extracted after 0h, 14h, 22h and 42h incubation. Culture filtrates (5ml) were combined with V8 agar (30%; 15ml) and inoculated with a plug of *P. infestans*. Mycelial growth was measured after 14 days. Points are means of three replications with standard error bars. The control was *P. infestans* grown on V8 (30%) agar only.

The culture filtrate of isolate 13 extracted at 22h inhibited *P. infestans* by approximately 27%, this decreased quite sharply to almost no inhibition just 8h later (Fig. 4.17).

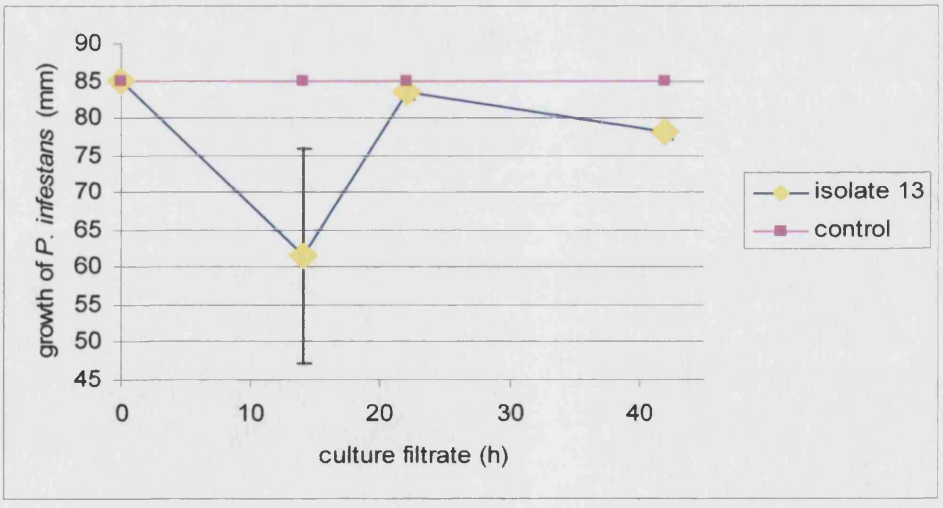

FIG. 4.17 Inhibitory activity of culture filtrates of isolate 13 extracted after 0h, 14h, 22h and 42h incubation. Culture filtrates (5ml) were combined with V8 agar (30%; 15ml) and inoculated with a plug of *P. infestans*. Mycelial growth was measured after 14 days. Points are means of three replications with standard error bars. The control was *P. infestans* grown on V8 (30%) agar only.

The culture filtrates of isolates 3, 6, 9 and 15 all demonstrated similar inhibitory patterns with highest suppression by 14h extracts (approximately 12 to 18% inhibition), decreasing at 22h (12 to 6% inhibition) with minimal inhibition evident by 42h extracts (Fig. 4.18).

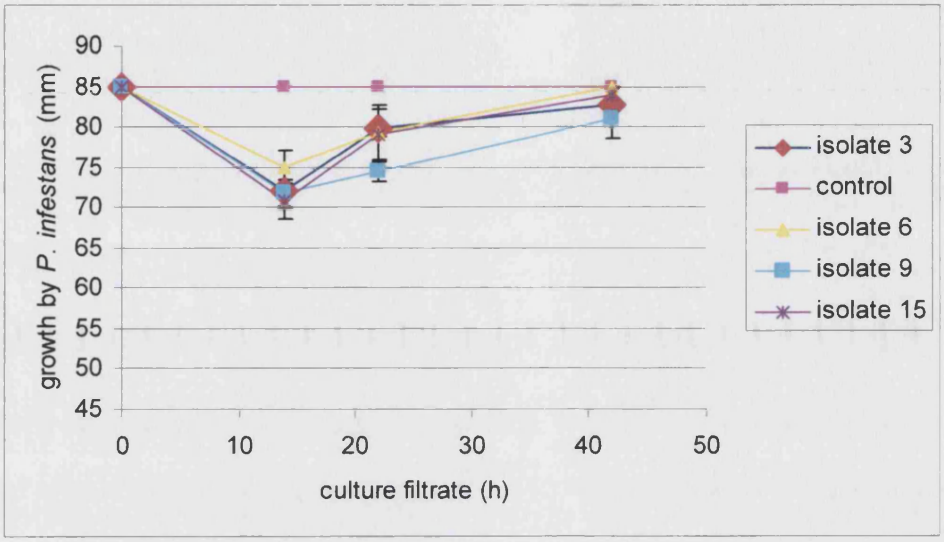

Fig. 4.18 Inhibitory activity of culture filtrates of isolates 3, 6, 9 and 15 extracted after 0h, 14h, 22h and 42h incubation. Culture filtrates (5ml) were combined with V8 agar (30%; 15ml) and inoculated with a plug of *P. infestans*. Mycelial growth was measured after 14 days. Points are means of three replications with standard error bars. The control was *P. infestans* grown on V8 (30%) agar only.

Isolate 14 exhibited a slightly different growth trend. The exponential growth phase was slightly slower reaching maximum growth at 50h (Fig. 4.19). Stationary phase was reached at a similar absorbance. Isolate 11 reached stationary phase at a lower absorbance (A=1.5 rather than over 2) also at 50h (Fig. 4.20). Samples of culture filtrate from isolate 8 and 14 were collected at 0h, 22h, 42h and 58h after inoculation. Isolate 12 reached stationary phase at approximately 20h but at a substantially decreased absorbance, A=0.5 (Fig. 4.21).

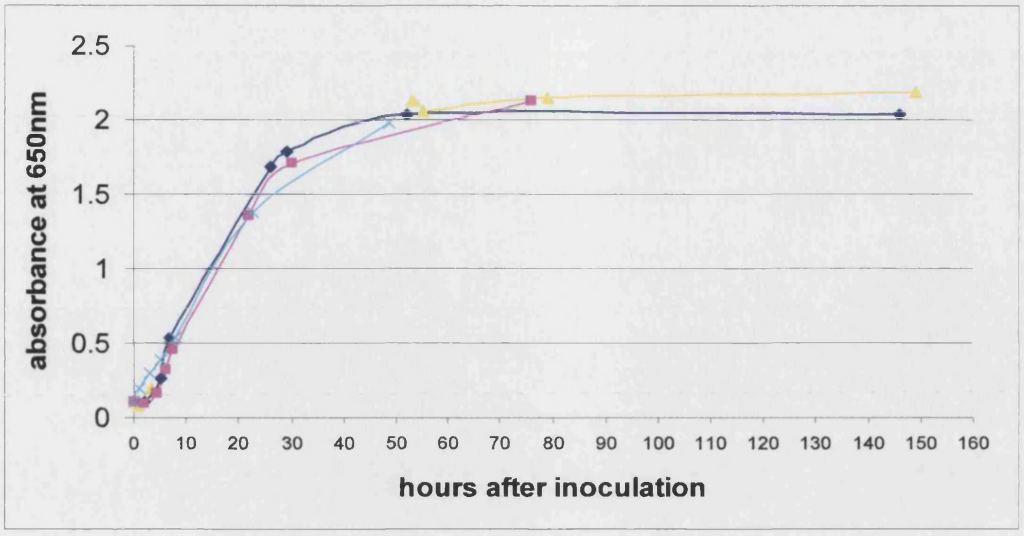

FIG. 4.19 Growth curve of isolate 14. The experiment was repeated seven times and from this data growth curves were constructed. Trends with anomalous results were not included.

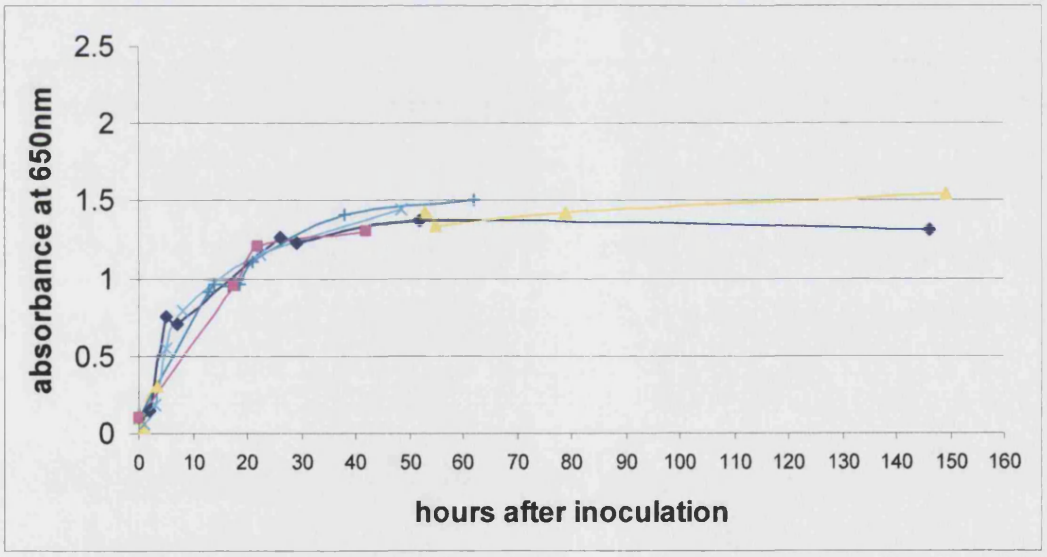

FIG. 4.20 Growth curve of isolate 11. The experiment was repeated seven times and from this data growth curves were constructed. Trends with anomalous results were not included.

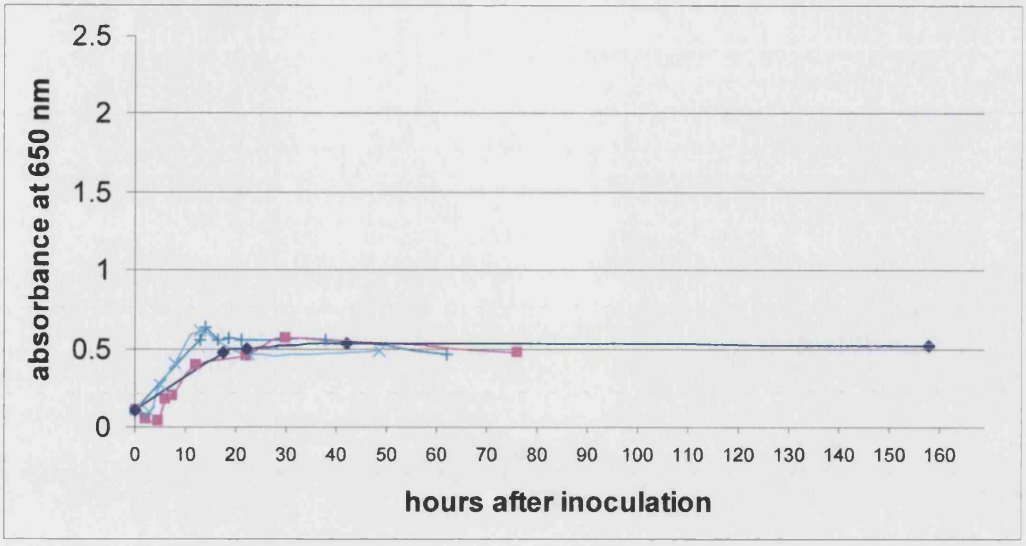

FIG. 4.21 Growth curve of isolate 12. Samples of culture filtrate were taken at 0h, 14h, 22h and 42h after inoculation.

Culture filtrates of isolates 11, 12 and 14 showed no inhibitory activity towards the mycelial growth of *P. infestans* (data not shown).

4.3.2 Visible interactions between the antagonists and *P. infestans*

Examination of the interface between *P. infestans* and substances diffusing from antagonists using the inverted microscope did not demonstrate clear effects except with isolate 7. In this case a clear zone of inhibition was observed between the pathogen and bacteria (Fig. 4.9). On examining this under the microscope the substance(s) were clearly affecting the hyphae morphologically which resulted in inhibited growth (Fig. 4.22). Fig. 4.23 illustrates hyphae on the same plate but at the edges of axis D (Fig. 4.1) and normal growth is observed.

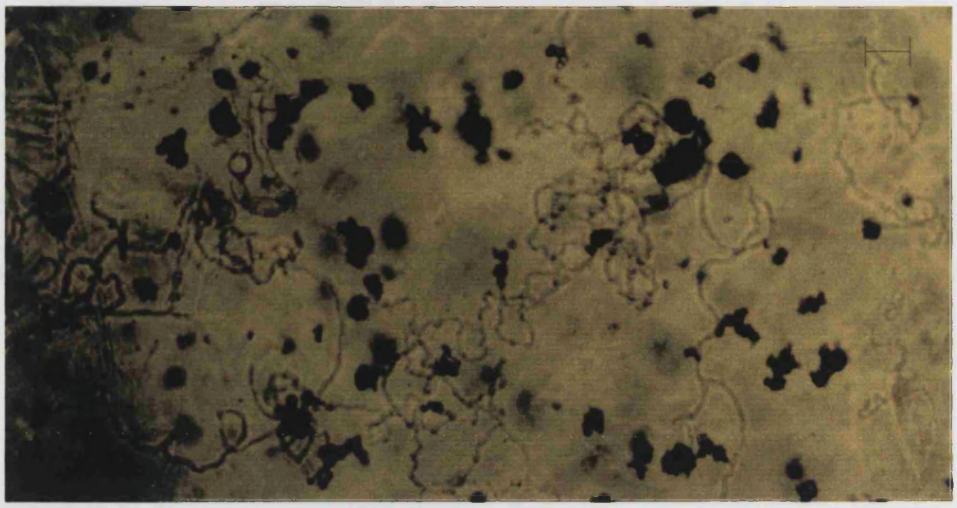

FIG. 4.22a *P. infestans* hyphae at edge of zone of inhibition (axis C, Fig. 4.1) when antagonised *in vitro* by isolate 7 (*Pantoea agglomerans*) using an inverted microscope. One bar corresponds to 100µm.

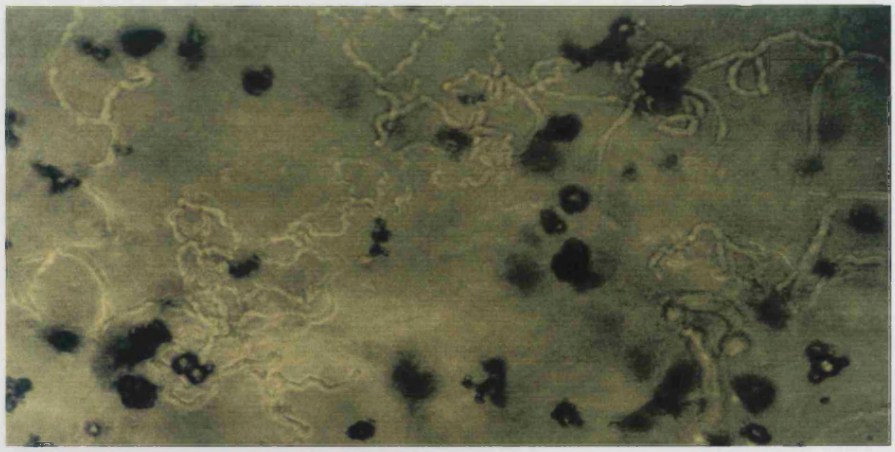

Fig. 4.22b Same image as Fig. 4.22a but at a higher magnification. One bar corresponds to 100 µm.

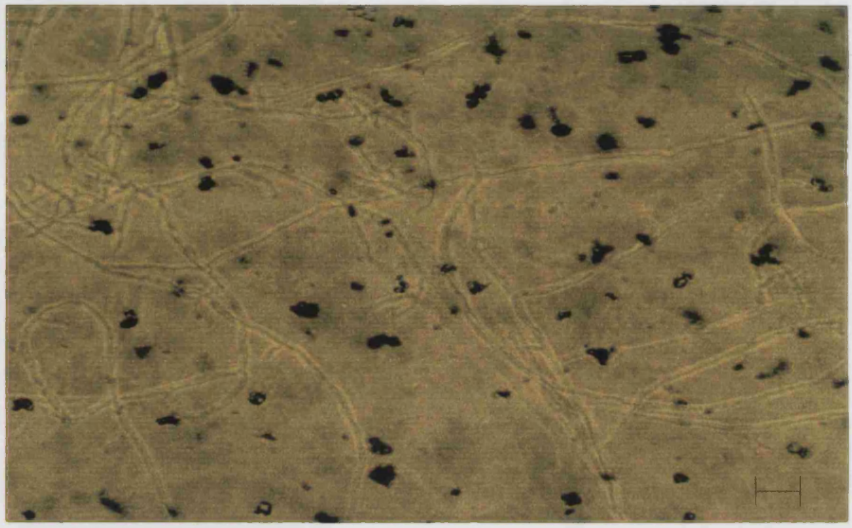

FIG. 4.23 *P. infestans* hyphae on same plate as Fig. 4.19 but at non-inhibited edges exhibiting normal growth. One bar corresponds to 100 µm.

Examination of plates where antagonists had been inoculated close to the *P. infestans* plug revealed clear hyphal colonisation by two *Pseudomonas putida* strains (isolates 1 and 3) and *Bacillus thuringiensis* (isolate 10; Fig. 4.24, 4.25, 4.26a and 4.26b). Colonisation by the other isolates was not observed.

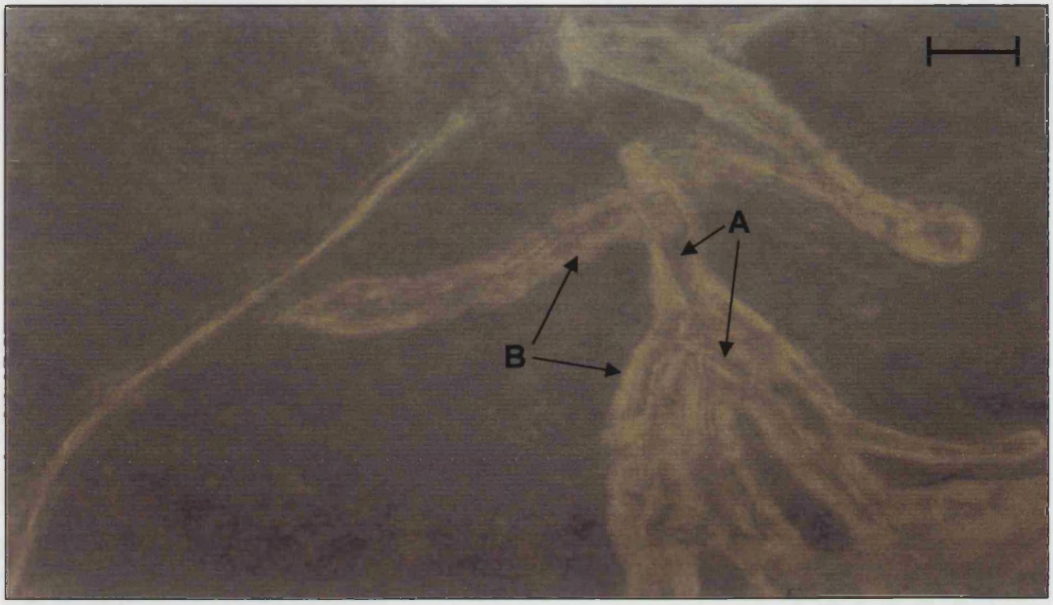

FIG. 4.24 Clear colonisation of *P. infestans* hyphae (A) by isolate 1, a strain of *Pseudomonas putida* (B) (inverted microscope). One bar corresponds to 100 µm.

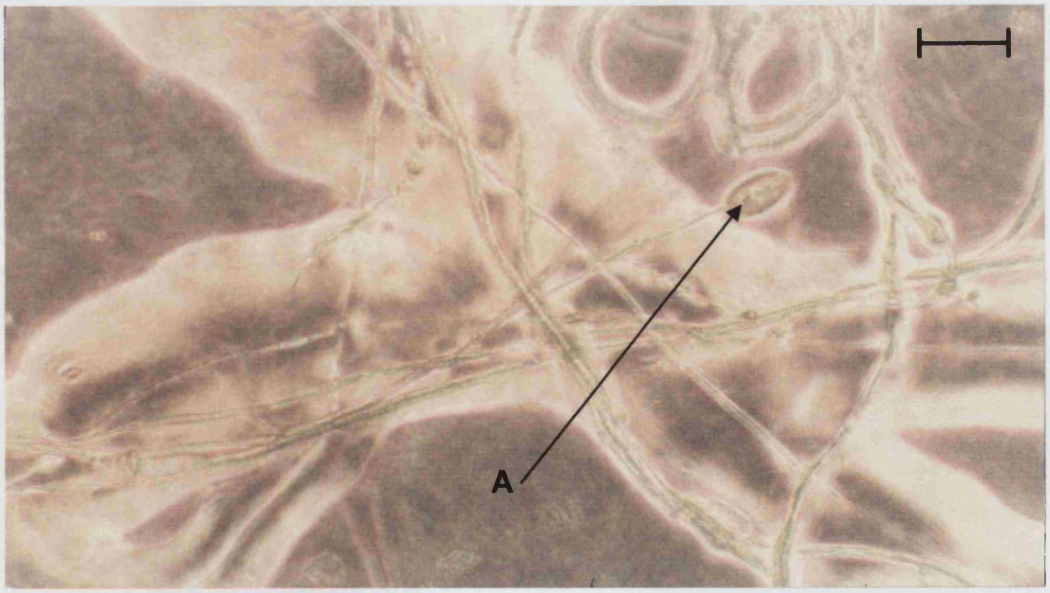

FIG. 4.25 Isolate 3, a strain of *P. putida* showing clear colonisation of *P. infestans* hyphae (inverted microscope). A bacterial halo surrounding a sporangium is evident in this picture (A). One bar corresponds to 100 µm.

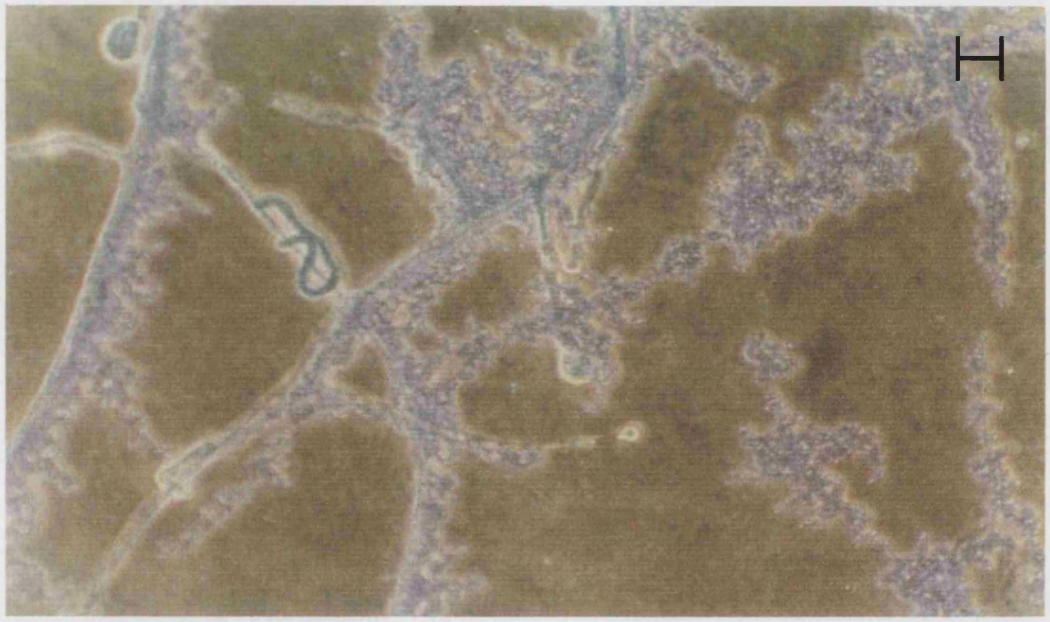

FIG. 4.26a Isolate 10, a strain of *Bacillus thuringiensis,* colonising *P. infestans* hyphae (inverted microscope). One bar corresponds to 100 µm.

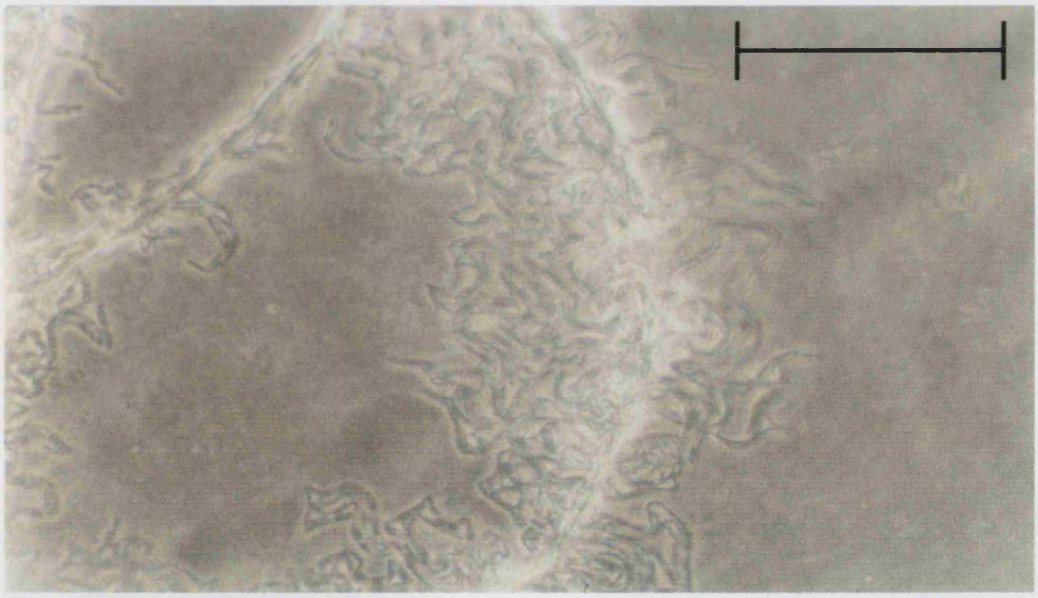

FIG. 4.26b Isolate 10, *B. thuringiensis* colonising *P. infestans* (inverted microscope). One bar corresponds to 100 µm.

4.3.3 Hyphal colonisation

When liquid was added to the column containing *P. infestans* mycelium it did not flow through even when substantial pressure was applied. It was therefore not possible to use this method to measure colonisation of *P. infestans* hyphae.

4.3.4 Siderophore production

4.3.4.1 Siderophore production in liquid medium (Scher and Baker, 1982)

The absorption spectra of the culture filtrates indicated peaks at approximately 400nm when isolates 1 and 10 were grown in low iron environments. The peaks were not evident when the isolates were grown in high iron environments. When the experiment was repeated, however, the results were not reproducible. It was observed that isolate 7, when grown in ß-1,3-glucanase inducing medium, was producing a yellow-green fluorescent compound visible to the naked eye. The absorption spectrum of the culture filtrate was measured revealing a peak at approximately 400nm (Fig. 4.27).

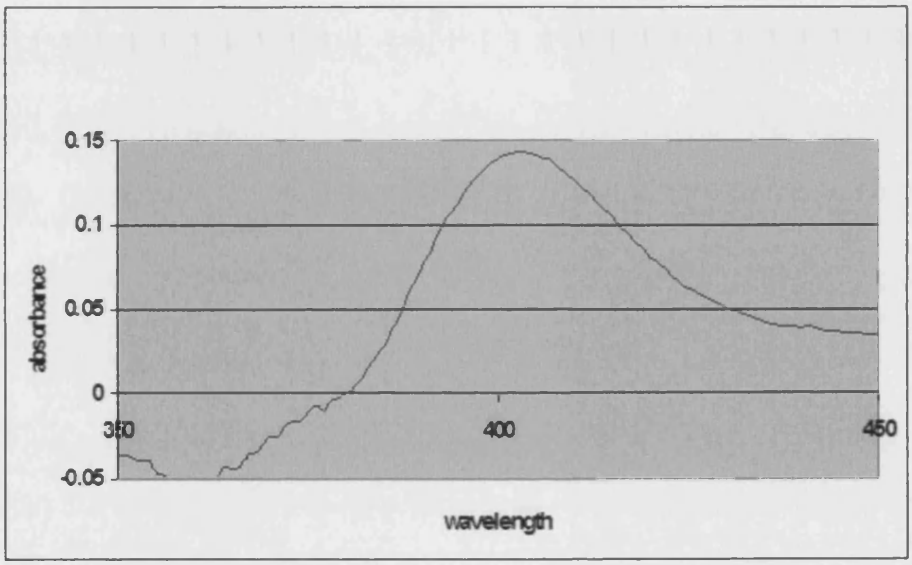

FIG. 4.27 Absorption spectrum of the culture filtrate of isolate 7 when grown in MLM with the carbon source replaced with *P. infestans* cells walls (1% w/v) and no trace iron. A clear peak is evident at approximately 400nm indicative of siderophore production.

4.3.4.2 CAS agar

Inconsistent results were obtained using the procedure of Schwyn and Neilands (1987) to prepare CAS agar. A range of colours was obtained from very pale blue to intense green.

4.3.4.3 Modified CAS agar

A consistent colour was obtained with production of the modified CAS agar on different occasions. Therefore siderophore production was measured on this medium. Table 4.3 summarises the colour change expressed as the maximum width of colour change at any point around the bacterial colony.

Table 4.3 Siderophore production of modified CAS agar

Isolate	Orange halo (mm)[a]	Growth[b]
1	6.00 ± 0.25	Good
2	7.00 ± 1.00	Good
3	16.25 ± 1.10	Good
4	2.25 ± 1.80	Good
5	7.75 ± 2.05	Good
6	5.50 ± 1.15	Good
7	7.25 ± 1.10	Good
8	8.75 ± 1.13	Good
9	5.25 ± 0.85	Good
10	4.40 ± 1.45	Retarded
11	5.25 ± 2.05	Good
12	4.50 ± 0.75	Good
13	0.50 ± 0.95	Slightly retarded
14	0	Retarded
15	0	Retarded

[a] The maximum width of the orange halo on the outer edge of the bacteria was measured. These results are the mean average of four repeats.

[b] Growth was determined as good corresponding to the same growth observed on nutrient agar. Slightly retarded referred to reasonable growth but some retardation evident, retarded referred to little or no growth on the agar.

4.3.4.4 Siderophore production in liquid medium (Alexander and Zuberer, 1991)

The use of deferoxamine mesylate (DFOM) to determine a standard curve in order to calculate siderophore production was effective (Fig. 4.28).

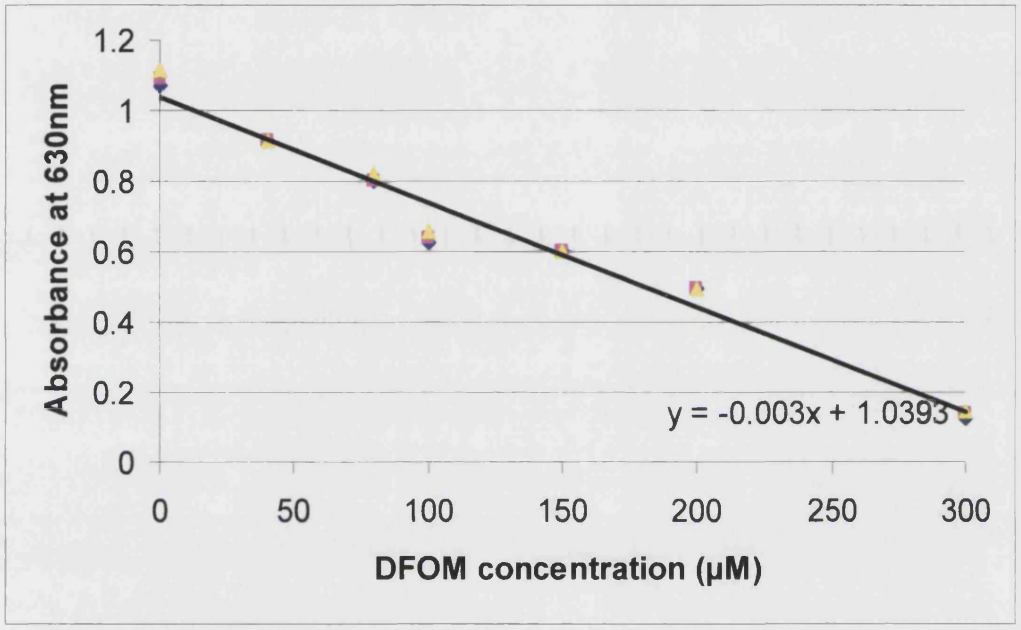

FIG. 4.28 Standard curve of the iron chelator, DFOM, for determination of siderophore concentration.

At concentrations of DFOM above 300µM the dye became bleached and was no longer sensitive to increasing concentrations (Fig. 4.29). In order to measure absorbance at these concentrations it would therefore be necessary to increase the proportion of dye in the reaction mixture.

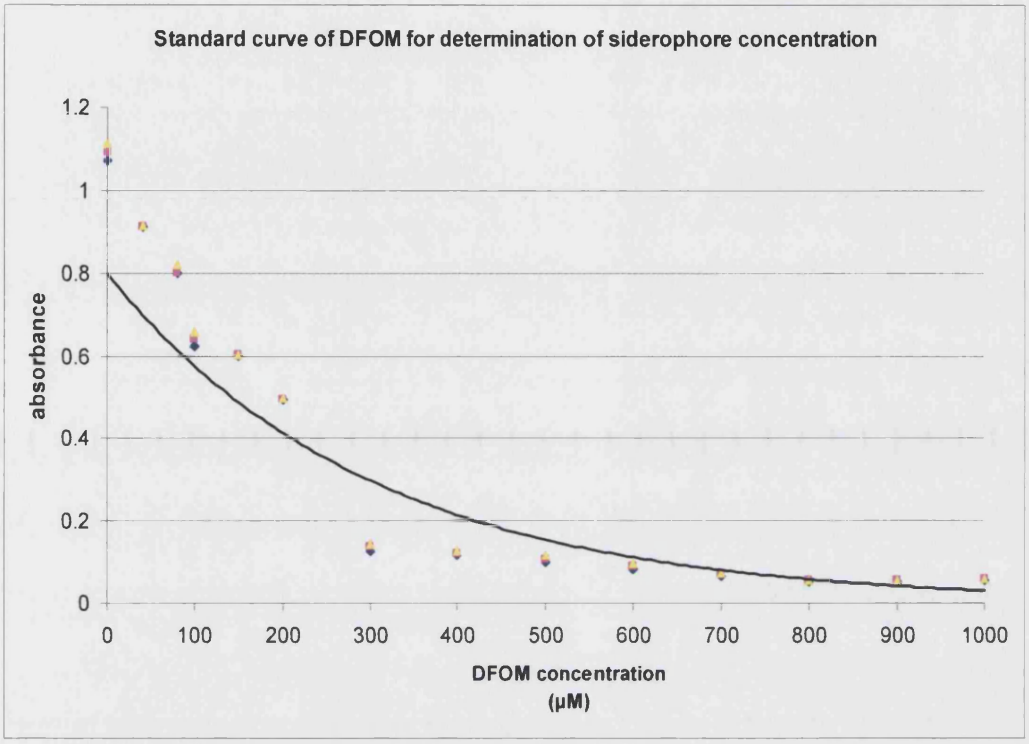

FIG. 4.29 Standard curve of DFOM with bleaching evident at over 300µM.

Only four of the isolates displayed no siderophore production by this method. Eight of the isolates produced over 150µM siderophore as determined by the DFOM standard curve (Fig. 4.30).

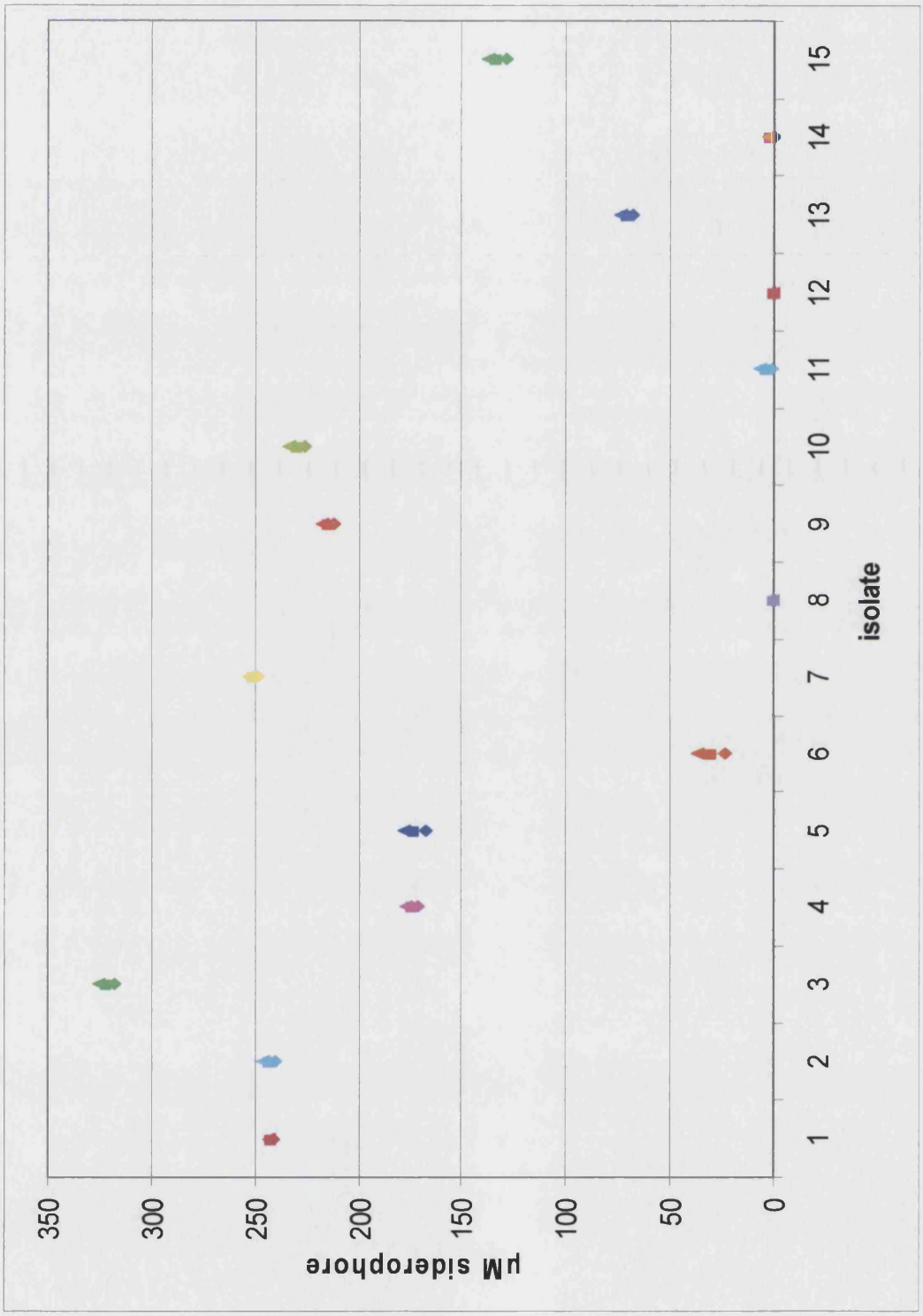

FIG. 4.30 Graph illustrating production of siderophores by potential BCAs as determined by the DFOM standard curve. Each point is the mean average of six replicates with standard error bars.

The absorption spectra of the ferric siderophore complexes from different isolates showed some substantial variation in both the wavelengths at which they absorbed and the intensity of absorbance (Table 4.4). In order to ensure, where possible, that absorbance readings did not exceed one, culture filtrates were diluted by x4.

Table 4.4 Absorption peaks and colours of culture filtrates from antagonists grown in low iron environments and assayed with CAS dye.

Isolate	Absorption peaks (nm)	Colour of ferric siderophore complex
1 (Fig. 4.31) *Pseudomonas putida* strain	250-260 315 400	Brick red
2 (Fig. 4.32) *Pseudomonas putida* strain	250-260 400	Brown
3 (Fig. 4.33) *Pseudomonas putida* strain	250-260 400	Red
4 *Enterobacter amnigenus*	250-260 315	Purple
5 *Pantoea agglomerans* strain	250-260 320-330	Dark red
6 *Pantoea agglomerans* strain	250-260 320-330	Dark red
7 (Fig. 4.34) *Pantoea agglomerans* strain	250-260	Dark red
8 *Pantoea agglomerans*	250-260	No colour change
9 *Enterobacter* spp.	250-260 320-330	Red
10 (Fig. 4.35) *Bacillus thuringiensis*	250-260 320-330	Dark red
11 *Citrobacter gillenii* strain	250-260	No colour change
12 *Buttiauxella noackiae* strain	250-260	No colour change
13 (Fig. 4.36) *Trichosporon laibachii*	250-260 320-330	Dark red
14 *Trichosporon cutaneum* strain	250-260	No colour change
15 *Geotrichum candidum* strain	250-260 320-330	Red

The absorption spectrum of isolate 1 (*Pseudomonas putida* strain), which has a high siderophore production (242.05μM, Fig. 4.30), exhibited three absorption peaks (Table 4.4, Fig. 4.31).

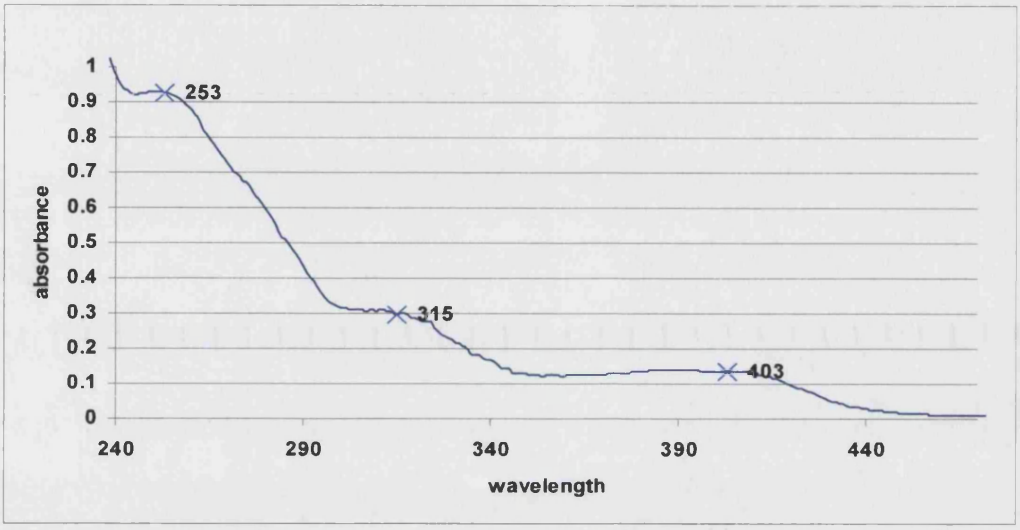

FIG. 4.31 Absorption spectrum of the culture filtrate of isolate 1 containing siderophores.

Isolates 2 and 3 (*Pseudomonas putida* strains) both produce high levels of siderophores (243.44 and 321.77μM respectively, Fig. 4.30). The initial concentration recorded for isolate 3 using an equal volume of indicator dye to culture filtrate was 272.87μM. The level of siderophore production on modified CAS agar indicated a much higher concentration compared to all other isolates which was not reflected in the liquid culture method. This suggested that the dye may have become bleached as seen in the standard curve and for this reason the culture filtrate was retested with 50% more dye solution. The absorption spectrum of isolate 2 has a definite shoulder at approximately 260nm but of fairly low intensity (A = 0.5) compared to the other isolates. Another much smaller peak is evident at 400nm (Table 4.4, Fig. 4.32).

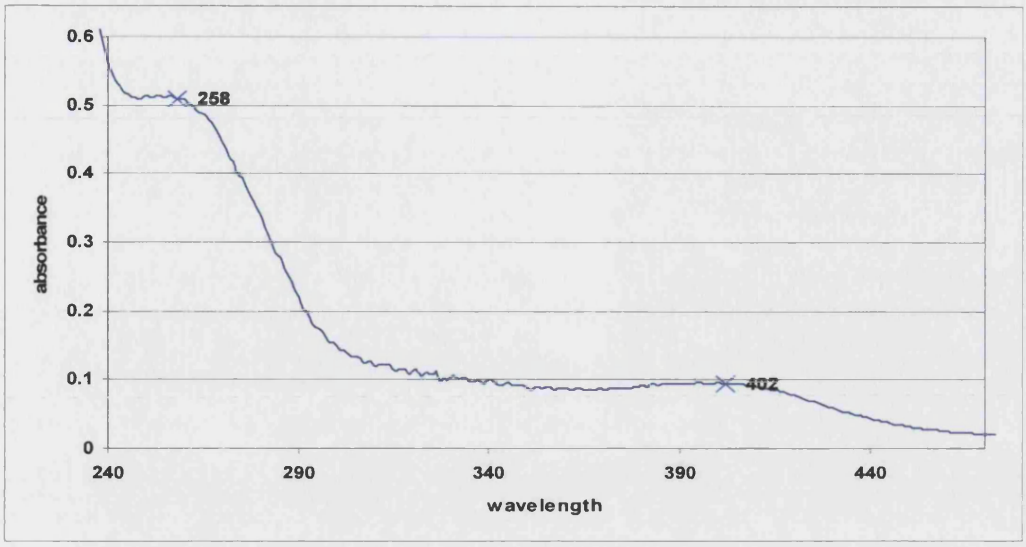

FIG. 4.32 Absorption spectrum of the culture filtrate of isolate 2 grown in medium inducing siderophore production.

The absorption spectrum of isolate 3 follows a similar pattern except with a much bigger peak at 400nm (Fig. 4.33).

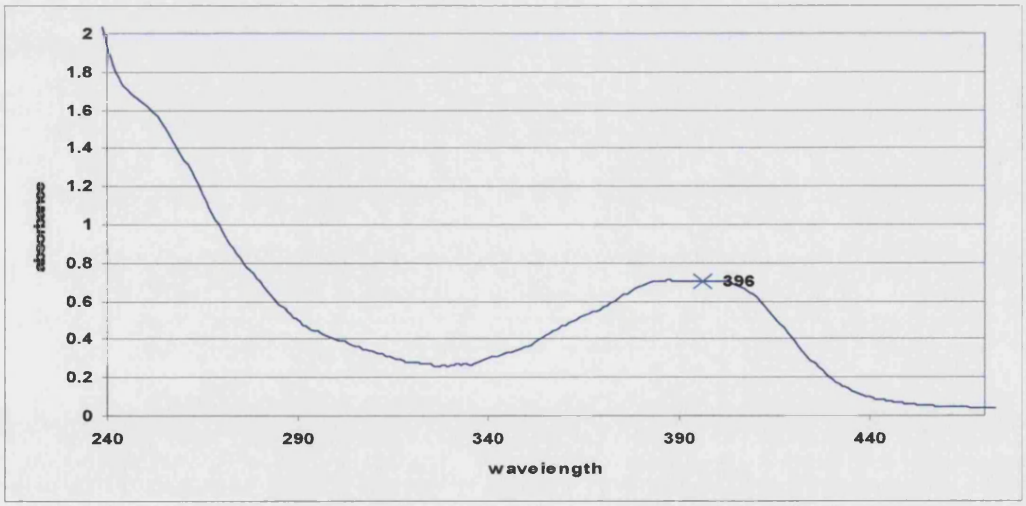

FIG. 4.33 Absorption spectrum of the culture filtrate of isolate 3 with a distinct peak at approximately 400nm.

Isolate 4 (*Enterobacter amnigenus)*, which had a lower but still considerable siderophore production of 174.05μM (Fig. 4.30), had a similar absorption spectrum to isolate 1 except there was no peak at 400nm and a lower absorption intensity at the other peaks (Table 4.4).

Isolate 13 (*Trichosporon laibachii*) produced a still lower concentration of siderophores (70.91µM, Fig. 4.30) with a similar absorption spectrum to isolate 4 except a more distinct peak occurred at 260nm (Fig. 4.34, Table 4.4).

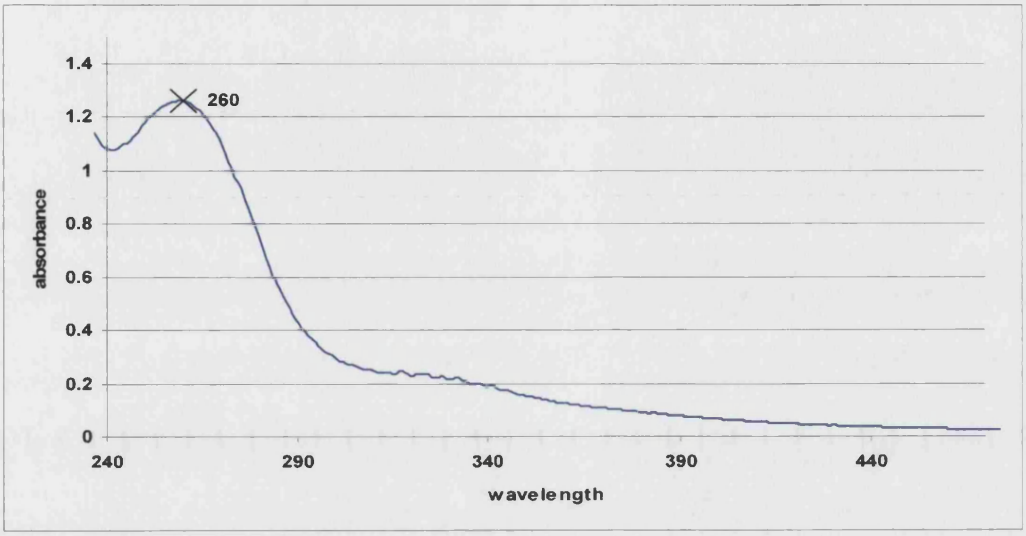

FIG. 4.34 Absorption spectrum of the culture filtrate of isolate 13 containing 70.91µM siderophores (Fig. 4.30).

Isolate 10 (*Bacillus thuringiensis* strain) displayed a very similar absorption spectrum to isolate 4, 6 and 13, with intense absorbance at 250 to 260nm and another peak at 320 to 330 (Fig. 4.35, Table 4.4) but which much higher siderophore production (229.65µM).

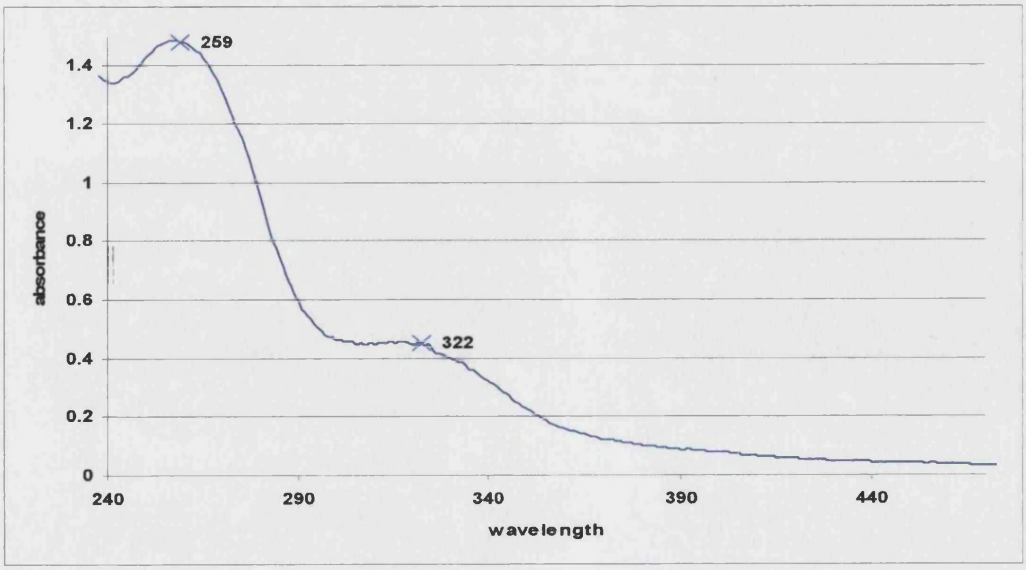

FIG. 4.35 Absorption spectrum of the culture filtrate of isolate 10 containing siderophores.

The absorption spectrum of isolate 5 is identical to that of isolate 6 (both *Pantoea agglomerans* strains) with a peak at 260 and 326nm, however siderophore production by isolate 5 is considerably higher (172.82µM compared to the 30.25µM by isolate 6, Fig. 4.30, Table 4.4). The absorption spectra of isolates 9 (*Enterobacter* spp., 214.87µM siderophore) and 15 (*Geotrichum candidum,* 132.91µM siderophore) are also comparable with less pronounced peaks at the same wavelengths (Table 4.4).

Isolates 7, 8 (both *Pantoea agglomerans* strains), 11 (*Citrobacter gillenii*), 12 (*Buttiauxella noackiae*) and 14 (*Trichosporon cutaneum*) with a siderophore production of 251.03µM, 0, 4.12µM, 0 and 1.63µM, respectively (Fig. 4.30), had very similar absorption wavelengths (Table 4.4). The absorption spectrum of the culture filtrate of isolate 7 (*Pantoea agglomerans* strain) is surprising given the high siderophore concentration measured and is in contrast to the peak obtained when grown in ß-1,3-glucanase inducing medium (Fig. 4.27). A small peak is evident at approximately 260nm with marginal absorbance at other wavelengths (Fig. 4.36). The peak was higher for isolates 8 and 11 (approximately A =1.2, Table 4.4).

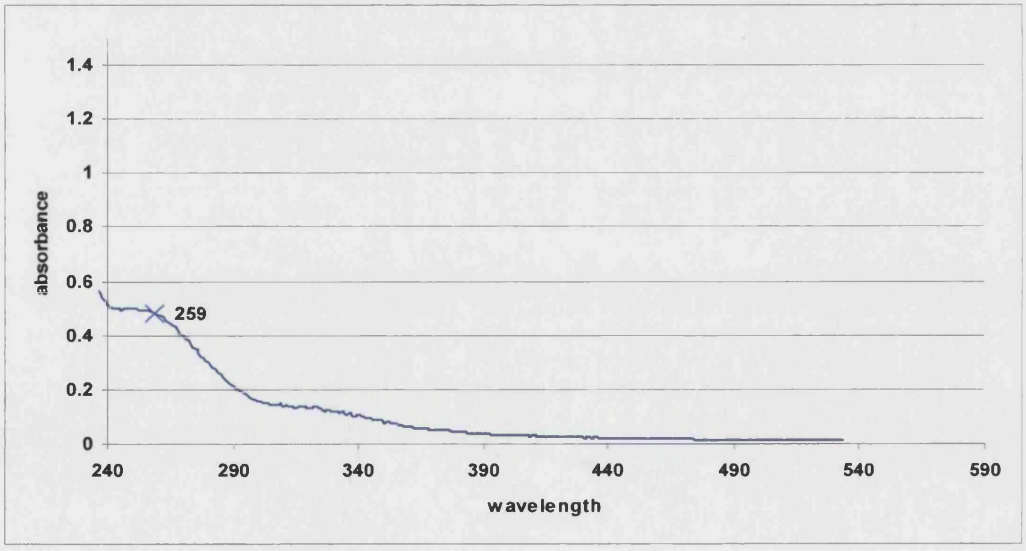

FIG. 4.36 Absorption spectrum of the culture filtrate of isolate 7. This culture filtrate contains high levels of siderophores (Fig. 4.30).

4.3.4 Indoleacetic acid production

Isolates 5, 7, 8 and 9 (all *Enterobacter* spp.) were positive for IAA production. Growth of *P. infestans* on medium containing IAA (20µM) at 14d was 68.7mm ± 0.6mm. The control (*P. infestans* only) grew 85mm with no deviation over the same time period.

4.3.6 ß-1,3-glucanase activity

Laminarin was used as the substrate and the glucose concentration measured by two methods.

4.3.6.1 Alkaline copper method (adapted from Nelson, 1944)

It was possible to calculate a standard glucose curve using the method of Dygert *et al.,* (1965) (Fig. 4.37), however inconsistent results were obtained when assaying culture filtrates. On cooling the reaction mixture a precipitate formed which did not redissolve on warming to room temperature. Various amounts of the reagents (Table 4.1) were tested but a precipitate always formed resulting in inconsistent results. However, this method indicated that isolates 2, 5, 6, 7, 8, 9, 10 and 11 were ß-1,3-glucanase producers.

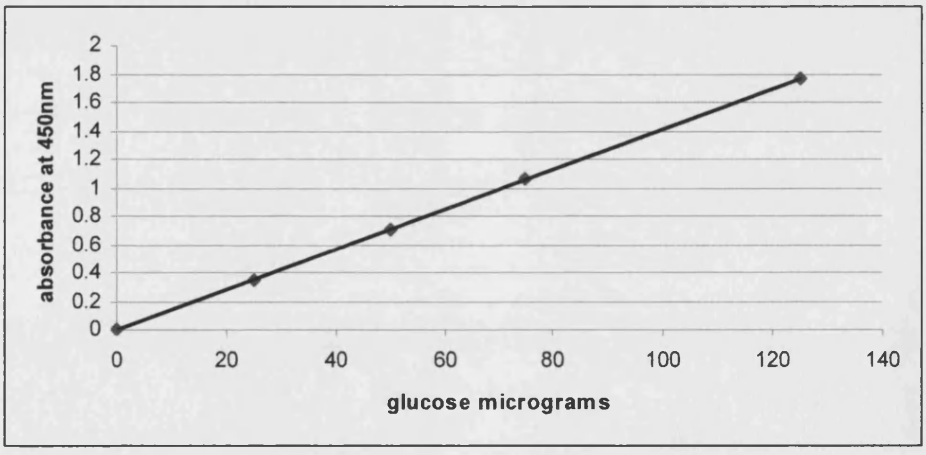

FIG. 4.37 Standard curve of glucose calculated using Dygerts *et al.*, (1965) method.

4.3.6.2 Glucose oxidase

A standard curve was determined using glucose (Fig. 4.38).

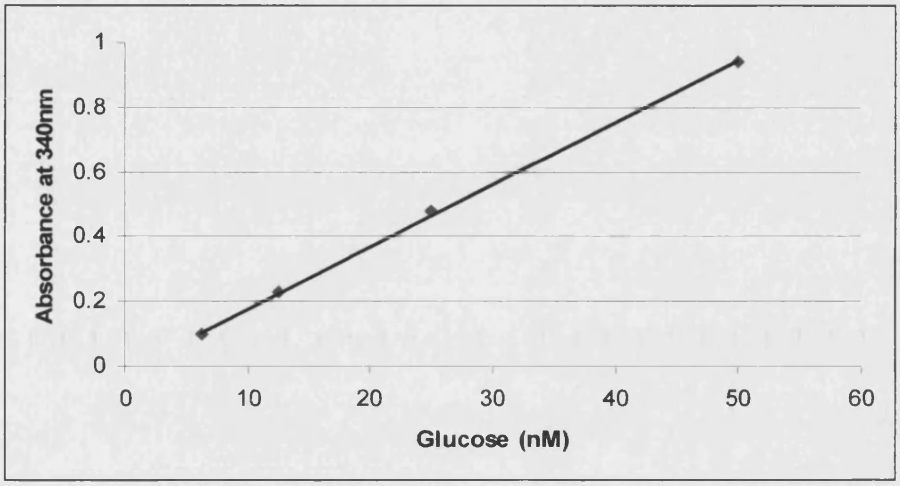

Fig. 4.38 Standard curve of glucose using glucose oxidase method.

This method was consistent but unexpected results were obtained. All isolate samples 0h, 48h and 96h contained lower levels of glucose than the control. All 168h samples except isolate 6 were less than 20.03nM, with the control containing 7.47nM. Isolate 6 contained 146.17nM glucose.

4.3.7. Protease activity

A standard curve was determined using proteinase K (Fig. 4.39).

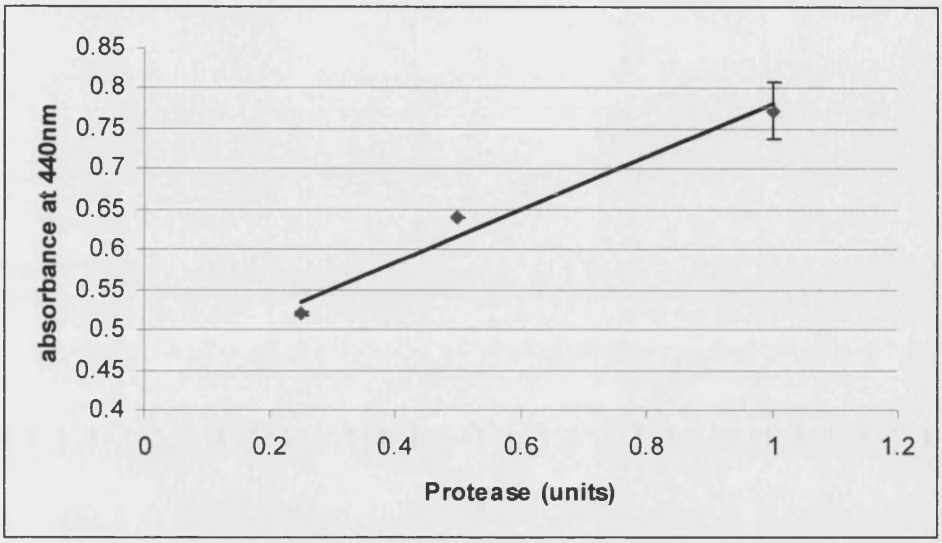

FIG. 4.39 Standard curve of proteinase K. Points are a mean of three replications with standard error bars.

None of the isolate samples were significantly different from the control.

Samples from isolate 1 and 10 (48h, 96h and 168h) were positive for proteolytic activity as determined by skimmed milk agar (clear agar indicates a positive result). All 0h and all isolate 13 and 14 samples were negative.

4.3.8 Effect of enzymes on *P. infestans* sporangia

4.3.8.1 ß-1,3-glucanase samples

Isolates 2, 8 and 10 (168h) caused 100% cell death. No other samples affected sporangial viability.

4.3.8.2 Protease samples

Samples from isolates 1, 10 and 14 (the negative control) caused 100% cell death. The control and isolate 13 did not affect cell viability (Fig. 4.40).

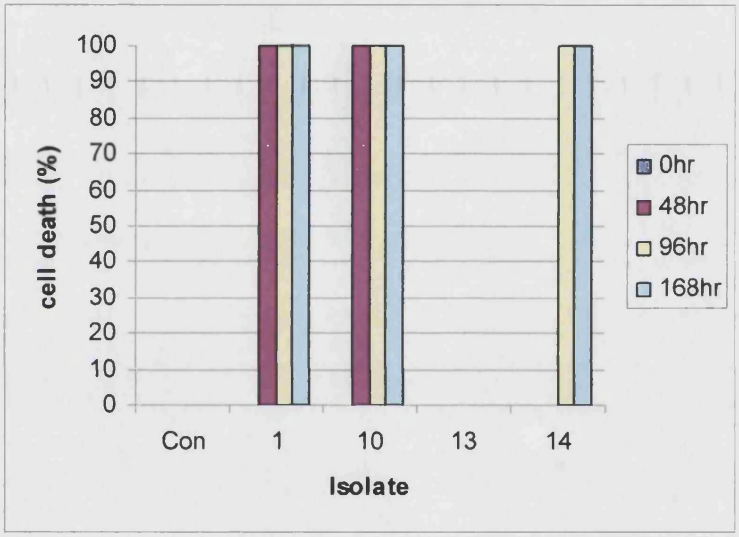

FIG. 4.40 Affect of protease samples on *P. infestans* sporangia viability. Controls were sterile medium used for inducing protease activity by antagonists. The experiment was repeated once.

Cell death was the same as the above when proteinase inhibitor was added to the enzyme extract and sporangia suspension. In addition all control extracts (sterile protease inducing media) and proteinase inhibitor killed sporangia. Sporangia incubated with proteinase inhibitor alone did not die.

When it was not possible to determine cell viability by fluorescence under the microscope observing the plate with the naked eye could discern between 100% and 0% cell viability as dead cells release esterases which cleave the fluorescein from the FDA resulting in fluorescence of the entire well contents.

4.3.9 Induced Systemic Resistance

4.3.9.1 Method adapted from Simons *et al.*, (1996)

This method was prone to contamination owing to the large number of manipulations involved. It was also extremely time consuming. Stunted growth of potato explants was observed probably as a result of ethylene production. Maintaining a balance between sterility and adequate gaseous exchange proved an insolubie problem and therefore this method was abandoned.

4.3.9.2 Similar method using Magenta vessels

This approach was more successful as contamination, set up time and inhibition of plant growth by ethylene production were all reduced. However, after one to two weeks the medium began to dry out which retarded plant development.

4.3.9.3 Magenta vessels and layers of medium

Potato explants in this setup did not grow satisfactorily. The roots would not penetrate below the first layer and this appeared to retard growth as well as preventing any contact of the plant with the test bacteria. Fig. 4.41 illustrates the retarded development compared to normal tissue culture grown potato.

FIG. 4.41 Normal tissue culture of potato explants in MS medium (left) compared to stunted growth (right) of potato explants in layered MS medium.

4.3.9.4 Magenta vessels without layers of medium

Potato plantlets grew as well as the normal tissue cultured plantlets and were considered satisfactory for inoculation and determination of ISR. Contamination levels were, however, extremely high and sterile systems were obtained for isolates 1 and 6 and the control only.

After 2 weeks all plantlets from all treatments were completely covered in late blight lesions.

4.4 DISCUSSION

When studying antibiotic production *in vitro* it is important to note this characteristic can be heavily influenced by the medium (Shoda, 2000). While the combination of the antagonism on V8 media (**4.2.1.1**) and of the culture filtrate from potential BCAs grown in MLM (**4.2.1.2**), should provide a reasonable indication of antibiotic production, it is possible the isolates that show no activity *in vitro,* may do so *in vivo.* The vegetable V8 juice used in the medium provides a high iron environment and this and the supplemented iron would eliminate siderophore activity as a means of mycelial inhibition. It is possible that antagonistic activity observed between isolates and *P. infestans* on these plates is attributable to lytic enzymes.

The decreasing levels of inhibition of *P. infestans* displayed by most antagonists on media with increasing iron levels could be largely attributed to the higher growth rate of *P. infestans* in the presence of iron. This is useful because it provides a good indication of the strength of the *in vitro* antibiosis. Isolate 1 still displays zones of inhibition on the high iron medium (Fig. 4.6), suggesting antagonism by a reasonably strong antibiotic. This isolate is known to produce hydrogen cyanide (Table 3.2), which has been demonstrated to contribute to the ability of *Pseudomonas fluorescens* strain CHA0 to inhibit black root rot of tobacco (*Thielaviopsis basicola,* Handelsman and Stabb, 1996). Application of a BCA which operates predominantly by the production of HCN may not be advisable owing to the widespread occurrence of cyanide-resistant respiratory pathways in microorganisms. Such a selection pressure may result in the emergence of pathogens containing cyanide-resistant oxidases (Handelsman and Stabb, 1996). High siderophore production by this isolate is, however, also evident (Fig. 4.30). Extensive studies have been undertaken to elucidate the mechanism by which *Pseudomonas putida* inhibits plant diseases, most indicating that antagonism by antibiotics and siderophores predominates (Shoda, 2000, de Boer, 2003). The results obtained in this study suggest these modes of action contribute substantially to the inhibition of *P. infestans* by isolate 1. This isolate was also positive for production of a number of potential cell wall degrading enzymes which may be contributing to this iron independent *in vitro* antagonism (Table 3.2).

The very low levels of inhibition exhibited by isolates 2 and 3 on all V8 media (Fig. 4.3, 4.4, 4.5 and 4.13) and the high levels of siderophore production (Fig. 4.30) suggests iron competition as the predominant mechanism of antagonism.

On V8 medium only, isolate 7 inhibited *P. infestans* at 14 days to the same extent as 9 days, indicating that the antagonist was producing a compound that could permanently inhibit mycelial growth. *Pantoea agglomerans* (*Enterobacter agglomerans*) produces at least five different antibiotics effective against pathogens such as *Erwinia* spp., *Pseudomonas syringae* pv. tomato and *Klebsiella pneumoniae,* for example (Wright *et al.,* 2001). Comparison of normal *P. infestans* hyphae with hyphae at the edge of the zone of inhibition revealed a morphological difference (Fig. 4.22 and 4.23). Isolate 7 apparently produces a diffusible substance that severely kinks the pathogen hyphae, preventing further growth. This is unlikely to be as a result of lytic enzymes as there is no evidence of digestion of the pathogen cell wall. *Pantoea agglomerans* inhibits the causal agent of fire blight, *Erwinia amylovora,* by production of various pantocin antibiotics. *In vitro* inhibition of this pathogen by these antibiotics can be reversed by the incorporation of various amino acids into the medium (Wright *et al.,* 2001, Stockwell *et al.*, 2002). These amino acids did not, however, have any effect the inhibition of *P. infestans* by isolate 7, or any other *Pantoea agglomerans* strain (5, 6, 8 and 9). This may be because insufficient amino acid was incorporated into the medium (personal communication Wright, 2003) or that inhibition *in vitro* of *P. infestans* by isolate 7 is not caused solely or at all by pantocin antibiotics.

Pantoea agglomerans has also been observed to compete for nutrient and infection sites, parasitize other plant pathogens and inhibit them by producing acidic conditions (Nunes *et al.*, 2002). This last approach could be particularly effective as mycelial growth of *P. infestans* is inhibited on V8 agar with no calcium carbonate (data not shown). V8 juice has a low pH suggesting the development of *P. infestans* may be sensitive to acidic conditions.

Antagonism exhibited by all *Enterobacter* isolates (5 – 9) except 4 (*Enterobacter amnigenus*), suggest production of diffusible compounds, although to a lesser extent than isolate 7. Close examination of the zones of inhibition by isolate 5 and 7 suggests that the levels may be similar. However, isolate 7 has spread from the site of inoculation and therefore antagonises more successfully whereas

isolate 5 has not (Fig. 4.42). Isolate 9 was identified by 16S rDNA sequencing as having 99% similarity to an *Enterobacter* spp. which produces the antibiotic 2,4-diacetylphloroglucinol (DAPG, Picard *et al.*, 2000). This antibiotic may be responsible for the inhibition of *P. infestans* by isolate 9. The inhibition was not reversed by the incorporation of amino acids into the medium suggesting pantocin antibiotics are not responsible.

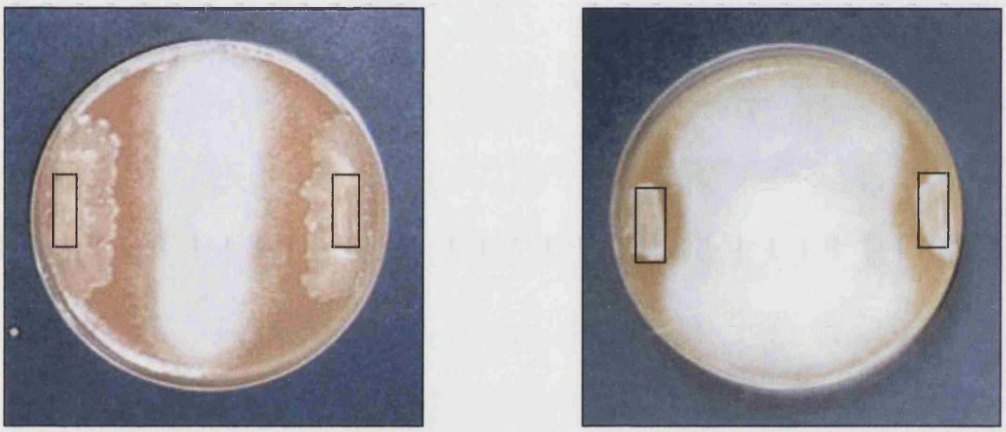

FIG. 4.42 Antagonism by both isolates 7 (left plate) and 5 (right plate), with spread from the point of inoculation by isolate 7, probably contributing to increased inhibition of *P. infestans*. Boxes represent the points of inoculation.

Isolates 4 and 12 showed very little evidence of inhibition *in vitro*. Isolate 4 displays reasonable siderophore production in liquid culture and on CAS agar however, isolate 12 induces some colour change on CAS agar but appears to produce no siderophores in liquid culture. These isolates are therefore likely to operate by alternative mechanisms such as competition, which may not be evident on high nutrient V8 or by ISR.

Isolates 13 and 15 did not appear to produce diffusible antagonistic compounds indicated by the absence of a zone of inhibition. However, substantially reduced growth of *P. infestans* is evident, possibly as a result of niche exclusion or competition. Isolates 10, 13 and 15 exhibit high levels of radial growth from the initial site of inoculation, much more so than other isolates (Fig. 4.11, 4.12 and 4.13), resulting in a large niche occupation. In a few instances zones of inhibition were evident for isolate 10 indicating that antibiotic production may be occurring on occasion and is very sensitive to the medium. *Bacillus thuringiensis* (strain

10), along with *B. cereus,* is known to produce the antibiotic zwittermycin A which affects the growth and activity of a range of plant pathogenic organisms, in particular *Phytophthora* and *Pythium* species (Raaijmakers *et al.*, 2002). In other instances inhibition by isolate 10 was similar to that by isolates 13 and 15.

Antibiotic activity in liquid culture is reliant on the growth stage of the producer (Eckwall and Schottel, 1997). To account for this it was important to determine the growth curve of each isolate so that each phase could be tested for antibiotic production. The growth rate may also be important when considering production economics and as an indication of the rate of growth in the plant rhizosphere which may influence disease control efficiency. Isolates 14 and particularly 12 have comparatively low growth levels in liquid culture (Fig. 4.19 and 4.21) which may be reflected in a reduced ability to control disease in the field and higher production costs. However, in certain instances high population densities are not required for efficient disease suppression. *Pseudomonas cepacia* and *Bacillus cereus* achieve only modest populations on roots of field-grown plants and appear to replace, not augment, the population of indigenous members of their species (Handelsman and Stabb, 1996). All *Pseudomonas* (1 to 3) and *Enterobacter* isolates (4 to 9) displayed very similar growth curves, quickly (20h) reaching stationary phase.

In agreement with the *in vitro* plate antagonism assays the culture filtrates of isolates 4 and 12 did not inhibit mycelial growth. In contrast to the *in vitro* antagonism assays the culture filtrates of isolates 5, 8, 10, 11 and 14 did not inhibit *P. infestans.* This may be due to sensitivity of antibiotic production to the medium. Eckwall and Schottel (1997) found antibiotic production in liquid culture by *Streptomyces diastatochromogenes,* which suppresses potato scab, peaked at the initial stages of the stationary phase. In agreement with this, culture filtrates of isolates 1, 2 and 7 displayed maximum inhibitory activity towards *P. infestans* at the start of the stationary phase (22h). Isolates 2, 6, 9, 13 and 15, however, exhibited maximum activity in the sample taken during the exponential growth phase (14h). These results suggest that isolates 13 and 15 are producing an antibiotic compound that may be contributing to *P. infestans* inhibition in addition to the niche exclusion observed on the *in vitro* antagonism plates (4.12 and 4.13). This method suggests that isolate 3 is producing an antibiotic compound (Fig. 4.18), unlike the results from the alternative method of measuring antibiotic production (Fig. 4.7). This organism may only produce the compound

during the exponential phase of growth and this was not achieved by culture on solid media. These results suggest that antibiotic production varies substantially with growth phase and with microorganism. Incorporation of the culture filtrates into the media may also have affected other aspects of growth of *P. infestans* such as sporangial production. It would have been useful to measure these parameters.

It was not possible to measure hyphal colonisation by the method used by Yang *et al.,* (1994). This may be because the hyphae of *P. infestans* were too gelatinous to achieve a flow through of buffer. Potential BCAs may have become lodged easily by friction and not necessarily by colonisation. It was possible to observe visible colonisation of the hyphae by three of the isolates using an inverted microscope (Fig. 4.24, 4.25 and 4.26). It is not possible to infer from this experiment to what extent, if any, this colonisation contributes to inhibition of *P. infestans.* It has, however, been demonstrated that the ability of *Enterobacter cloacae* to bind to the hyphae of *Pythium ultimum* is critical for disease suppression (Nelson *et al.*, 1986). Additionally Yang *et al.,* (1994) used colonising deficient mutants of *P. putida* and *P. fluorescens* to demonstrate that hyphal colonisation of *Phytophthora parasitica* was important for *in vitro* inhibition. More recently *in vivo* expression technology has been used to study the colonisation of *P. parasitica* by *P. putida* 06909. The use of *P. putida* clones with different genes fused to a pyrimidine biosynthesis marker gene has suggested that a putative outer membrane porin protein at the 3' end of a partially sequenced clone, together with the ABC transporter may be involved in the uptake of specific chemicals from the pathogen (Lee and Cooksey, 2000).

Comparison of the methods of measuring siderophore production indicates variation among results. The Scher and Baker (1982) method produced inconsistent results and suggested that only 3 of the 15 isolates produced siderophores. The inconsistency is probably due to sensitivity of siderophore production to the medium. The absorption spectrum of the culture filtrate of isolate 7 grown in MLM medium with *P. infestans* mycelium as the carbon source shows a distinct peak at approximately 400nm (Fig. 4.27) and was accompanied by a yellow-green fluorescent colour, suggesting a pyoverdine or pseudobactin. This is not evident in the absorption spectrum of the culture filtrate when the isolate is grown in modified M9 liquid medium (Fig. 4.34), although high levels of siderophore are measured in this medium (Fig. 4.30). This indicates that this

isolate may be capable of producing more than one type of siderophore and this is influenced by the environment. Several microbes are known to produce more than siderophore, and in many cases the siderophores may be produced differentially. That is a more powerful chelator may only be produced when the first less powerful chelator fails to provide enough iron to the cell (Guerinot, 1994). *Azotobacter vinelandii* produces a pyoverdin-type siderophore, azotobactin and two catechol-type siderophores, azotochelin and aminochelin. The production of these siderophores follows a sequential induction pattern. As iron becomes limiting (7μM Fe^{3+}) the catechol siderophores are produced co-ordinately, if the iron continues to be limiting (≤ 3μM Fe^{3+}), azotobactin is formed. In this case the lower-affinity catechol siderophores appeared to be efficient solubilisers of mineral iron sources, while azotobactin scavenged soluble iron (Sevinc and Page, 1992). Isolate 7 (*P. agglomerans* strain) grown in modified M9 medium with no added iron produced a more powerful siderophore, possibly an enterobactin (Fig. 4.43) or ferrioxamine siderophore (Deiss *et al.*, 1998), than when grown in a slightly higher iron medium (3.6 μM $FeSO_4 \cdot 7H_2O$ per litre). Under these conditions $FeSO_4$ is insoluble, and the presence of iron in this form may also have influenced the differential siderophore production.

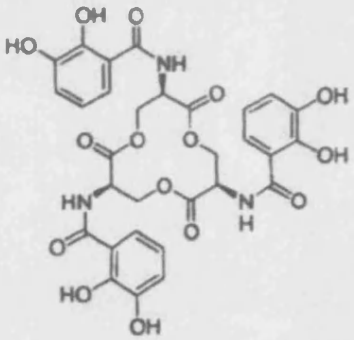

FIG. 4.43 Structure of enterobactin, a prototypical catechol type siderophore with the highest known binding constant, produced generally by enteric bacteria (Raymond *et al.*, 2003)

The inconsistent agar colour obtained on CAS agar (Schwyn and Neiland, 1987) is in agreement with other published work (Alexander and Zuberer, 1991).

Colours ranging from brown to green were obtained by these workers. This agar is therefore unsuitable for determining siderophore production.

To provide a quantitative indicator of siderophore production on agar maximum width of colour change was satisfactory. However, CAS-reaction rate (mm per day) may be preferable as this provides more information (Machuca and Milagres, 2003). At neutral pH (pH of CAS-blue agar and liquid medium) the monohydroxamate and trihydroxamate complexes are reddish-orange and orange coloured, respectively, while catechol complexes are reddish-purplish. Milagres *et al.*, (1999) suggest that colour change on CAS agar can be used as an indication of siderophore type. All colour changes on CAS agar in this investigation were to orange. In liquid medium three isolates (1, 2 and 3) produced brick red complexes suggesting monohydroxamate complexes. The remaining isolates (4, 5, 6, 7, 9, 10, 13 and 15) produced siderophores that formed purplish complexes, characteristic of catechols. Hydroxamate siderophores are very water soluble and considered to be more stable than catechol siderophores as they are less affected by pH fluctuations (Guerinot, 1994). The inconsistency of colour change, however, between solid and liquid medium suggests that this is not a reliable indication of siderophore type. The chrome azurol sulfonate assay is based on the colour change that accompanies transfer of the ferric iron from its intense (extinction coefficient of at least 100 000) blue complex to the siderophore. A detergent (HDTMA) must be present in order to achieve the intense colour, otherwise a charge-transfer extinction of a few thousand is realised (Neilands, 1995). Gram-negative bacteria are not affected by the detergent, which is, however, toxic to Gram-positive bacteria and fungi and therefore to isolates 10, 13, 14 and 15 in this investigation. It is therefore unlikely that siderophore production by these organisms was accurately indicated on this medium. Comparison with the results of these isolates from the liquid medium suggest that lower levels were recorded on the agar. Milagres *et al.,* (1999) modified the chrome azurol S agar plate assay so that the microorganisms have no direct contact with the dye and therefore are not inhibited by the detergent required for the assay. Agar plates are prepared with half the plate CAS agar and the other half the medium optimal for growth of the test organism. Consistent results were obtained for a range of organisms tested, suggesting that it is an appropriate method to avoid problems of inhibited growth.

Comparison of the results obtained for the other bacteria indicate some variability between the solid and liquid culture method. Siderophore production was higher on the agar for isolates 3, 6, 8, 11 and 12 (Table 4.3). Isolates 8 and 11 did not produce any detectable siderophores in liquid medium (Fig. 4.30). The liquid medium contained no added iron whereas the CAS agar contained the Fe-CAS indicator solution. Isolates 8 and 11 may produce low affinity siderophores which are capable of scavenging iron from the CAS-Fe^{3+} complex, but that are not capable of acquiring iron from a more iron limited environment (the modified M9 liquid medium) and are therefore not produced. Isolate 3 produced almost double the distance of colour change of any other isolate on CAS agar (Table 4.3) and also produced the most siderophore in liquid culture (Fig. 4.30). This could be as a result of the quantities of siderophores produced and the affinity for iron of the siderophores.

All three *P. putida* strains produced diffusible fluorescent pigments in liquid culture. These fluorescent yellow-green water-soluble siderophores are comprised of a quinoline moiety, responsible for the fluorescence, and a peptide chain of variable length bearing hydroxamic acid and α-hydroxy acid functions (Neilands, 1995). The absorption spectra of these three isolates (1, 2 and 3) were characteristic of the purified pseudobactin from plant growth promoting *P. putida* strain B10, with an absorption maximum in the visible near 400nm and a more intense absorption band in the ultraviolet range at 240nm, with a shoulder at 265nm (Alexander and Zuberer, 1991). Isolate 1 exhibited an additional peak at approximately 315nm (Fig. 4.31).

The complete structure of the pseudobactin from *P. putida* B10 was determined by single X-ray diffraction and spectroscopic methods. It consists of a linear hexapeptide in which the ornithine residue was cyclized into an *N*-hydroxy piperidone ring and the lysine residue linked to a fluorescent quinoline derivative (Teintze *et al.*, 1981, Fig. 4.45). The quinoline derivative is responsible for the peak at 400nm in the culture filtrate.

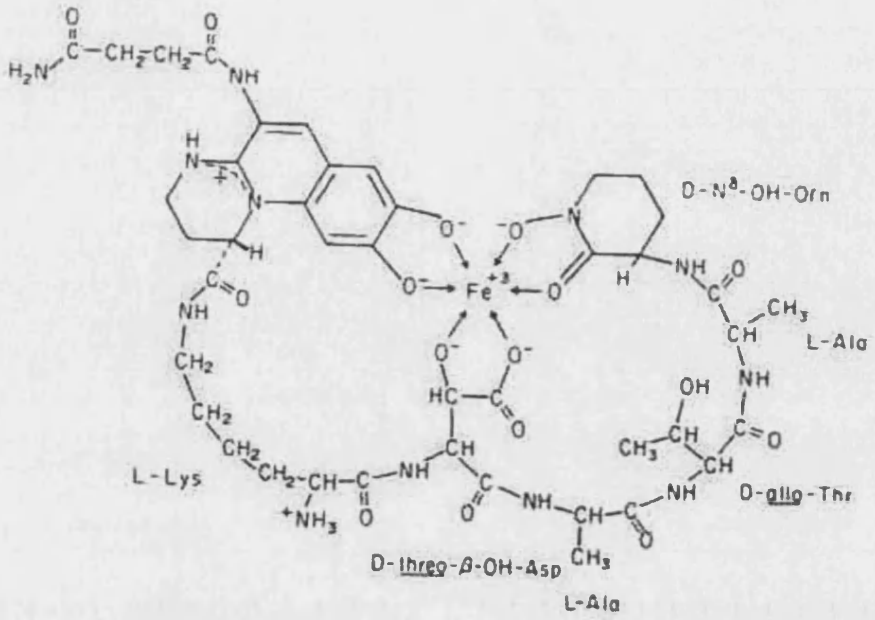

FIG. 4.44 Structure of the fluorescent siderophore, ferric pseudobactin (Strange, 1993)

Characterisation of other *Pseudomonas* spp. siderophores has revealed more variation than any other family of siderophores owing to differences in the number of amino acids in the peptide chain, their sequence and chirality. In all these siderophores, the hydroxyl acid from Asp residues, hydroxamate from ornithine and the *o*-dihydroxy group from the quinoline derivative form chelating groups for iron (Boopathi and Rao, 1999). Some *P. putida* strains have also been demonstrated to be capable of utilising siderophores in the rhizosphere produced by other bacterial species (Loper and Henkels, 1999), providing an additional competitive advantage.

As mentioned in chapter III isolate 1 fluoresced blue on KB under UV light (Fig. 3.2), suggesting the possibility of a *Pseudomonas aeruginosa* strain although the sequence data was more indicative of *P. putida*. *P. aeruginosa* produces three siderophores under iron limiting conditions; pyoverdine, pyochelin and salicylic acid (SA, Audenaert *et al.*, 2002). This latter compound, SA (or 2-hydroxybenzoic acid) has also been found to act as a siderophore in *P. fluorescens* and *P. cepacia* (Visca *et al.*, 1993). As well as functioning as an independent siderophore, SA is an intermediate in the biosynthesis of pyochelin in *P. aeruginosa* and of aeruginoic acid and pseudomonine produced by *P.*

fluorescens strains (Mercardo-Blanc *et al.,* 2001). SA fluoresces blue-purple under UV light suggesting a possible link with the fluorescence of isolate 1. It would be interesting to pursue this area especially considering the role of SA in inducing resistance within plants.

That the peak at approximately 260nm is present in the culture filtrates of isolates 8 and 11, both of which do not display any siderophore production suggesting that this peak is not necessarily associated with siderophores. There is a peak to some extent at approximately 320nm for all isolates exhibiting some level of siderophore production except 2 and 3 and no peak in the culture filtrates of those isolates with no siderophore production, suggesting that this wavelength is associated with some form of non-fluorescent iron chelating compound.

Antagonism related to siderophore-mediated iron competition may also have an indirect effect, for example, by enhancing the antagonistic effect of non-pathogenic *Fusarium oxysporum* by making the pathogen more susceptible to fungal competition for carbon (Lemanceau and Albouvette, 1993). In addition production of siderophores does not necessarily implicate a role at all for them in disease suppression. Paulitz and Loper (1991) generated fluorescent siderophore [pyoverdine] biosynthesis mutants of *P. putida.* Unlike the parental strain this mutant did not inhibit hyphal growth of *Pythium ultimum,* but protected cucumber seedlings from *Pythium* damping off in three different agricultural soils at levels statistically indistinguishable from the parental strain. This suggests that the siderophore was not critical for disease control.

IAA is the most abundant and widespread auxin, produced by approximately 80% of bacteria isolated from the rhizosphere (Dobbelaere *et al.,* 2003). It is a plant growth regulator which mediates an enormous range of developmental and growth responses (Noel *et al.,* 2001). The impact of IAA can range from beneficial to negative effects. Xie *et al.,* (1996) demonstrated beneficial root morphogenesis effects by a strain of *Pseudomonas putida* producing IAA. A mutant engineered to produce four times the amount of IAA had a deleterious effect. The biosynthetic pathway used by the bacteria to produce IAA may affect whether plant growth is promoted or inhibited (Persello-Cartieaux *et al.*, 2003). The isolates in this investigation that were positive for IAA production were identified as *Enterobacter / Pantoea agglomerans* (isolates 5, 6 and 8), synonymous with *Erwinia herbicola*, and an *Enterobacter* species (9) probably *E.*

agglomerans. *E. herbicola* has been demonstrated to produce IAA via indole-3-pyruvic acid and indole-3-acetaldehyde, the pathway which also predominates in higher plants and in contrast to the indoleacetamide pathway, believed to be a specific microbial IAA biosynthesis route utilised by phytopathogens such as *Agrobacterium tumefaciens* (Persello-Cartieaux *et al.*, 2003). The mechanisms by which one pathway results in beneficial growth and another pathway in inhibited growth are not fully understood. However, the isolates in this investigation probably produce IAA by the beneficial pathway and so would be likely to promote plant development and therefore provide an additional advantage to disease protection.

Noel *et al.*, (2001) examined the effect of IAA on the potato-*P. infestans* interaction and pathogen growth *in vitro*. They found that treatment of an excised potato leaflet with 10µM IAA resulted in a 50% reduction of disease severity and a 60% decrease in *P. infestans* ß-1,3-glucanase activity. A 45% growth inhibition of *P. infestans* mycelia was observed on V8-agar medium containing 1µM IAA which was overcome when external ß-1,3-glucanase was supplied. This led the authors to postulate a putative role for auxins in the potato-*P, infestans* patho-system as natural defence for pathogen spread and disease development. Inhibition of mycelial growth was also observed in this investigation when 20µM IAA was incorporated into the medium. It is possible that the production of IAA by isolates 5, 7, 8, and 9 is contributing to the mode of inhibition both *in vitro* and *in vivo*.

Chitinase activity was not measured in this investigation for the reasons mentioned in the introduction to this chapter. However, chitin derivatives can both inhibit the growth of several plant pathogenic fungi and induce plant defence mechanisms (Kendra and Hadwiger, 1984). Biphasic chitin waste-based composts, prepared by composting peat moss, sawdust and chitinous waste or cow manure until termination of the thermophilic phase and then amending with shrimp waste inducing a second thermophilic phase, reduced the growth of two oomycete plant pathogens, *Phytophthora fragariae* var. *rubi* and *Pythium ultimum*. The authors suggest that the inhibition of *Phytophthora* during the first phase was due to the presence of oligomeric chitin, while in the second phase was due to a proliferation of Gram-positive bacteria antagonistic to oomycete plant pathogens (Labrie *et al.*, 2001). Therefore it is possible that chitinase producing microorgansims in a high chitin environment may antagonise *P.*

infestans by the release of inhibitory chitin derivatives. As neither the potato tuber nor V8 agar are sources of chitin, inhibition observed in these environments is unlikely to be as a result of chitinase activity.

The chromogenic substrate, azoalbumin, had been used successfully to measure activity by protease K (Mischke, 1996) but was not suitable for measuring protease production by the bacterial isolates in this investigation. This may be because the bacterial proteases are more specific than protease K and that azoalbumin is not an appropriate substrate.

Proteolytic activity is probably contributing to the antagonism by isolates 1 and 10 as 100% cell death of sporangia is achieved with enzyme extracts exhibiting proteolytic activity. The control extract (sterile proteolytic inducing liquid medium) did not cause cell death. The negative control (isolate 14, 96h and 168h extracts) did, however, cause 100% cell death but was negative for proteolytic activity on skimmed milk agar. This suggests that this isolate was producing a non-protease anti-oomycetic compound.

The addition of the proteinase inhibitor did not reverse the activity of any of the extracts. This could be because the component in the extract responsible for killing the sporangia was not proteolytic or because the proteinase inhibitor was specific and did not affect the proteinase produced by the antagonist. Surprisingly the proteinase inhibitor added to the control and negative control extracts caused cell death while cell death did not occur when proteinase inhibitor alone was added to sporangia. The proteinase inhibitor combined with some constituent of the protease inducing media must have had an effect, such as a pH change, which caused cell death.

The presence of ß-1,3-glucanase activity in samples taken from cultures at 0h inoculation suggests a source of error. *P. infestans* cell walls were used as the sole carbon source in the medium used for inducing ß-1,3-glucanase activity. High levels of activity by the same enzyme have been detected in *P. infestans* (Noel *et al.,* 2001) and residues may have been transferred with the cell walls. A medium using laminarin as the sole carbon source may be more reliable. Fridlender *et al.,* (1993) studied ß-1,3-glucanase production by a *Pseudomonas cepacia* biocontrol strain and found that specific activity obtained with laminarin was twice that recorded when cell walls of *Rhizoctonia solani* were the carbon

source and much lower with cell walls of *Sclerotium rolfsii.* No activity was found in the presence of *Fusarium oxysporum* f. sp. *vasinfectium* and *Fusarium oxysporum* f. sp. *melonis* cell walls. The biocontrol strain was demonstrated to control *R. solani* and *S. rolfsii* successfully but neither *Fusarium* species. The lack of consistent results obtained in this investigation indicates that either none of the selected BCAs suppress *P. infestans* by the production of ß-1,3-glucanases or *P. infestans* cell walls are not effective inducers of ß-1,3-glucanase activity or both.

There have been some preliminary studies indicating the potential of ISR as a biocontrol mechanism effective against oomycete pathogens. Inoculation of tomato with two plant growth promoting bacteria *Bacillus pumilis* and *Pseudomonas fluorescens* elicited systemic protection against late blight to a level equivalent to systemic acquired resistance induced by *P. infestans.* This resistance significantly reduced zoospore and sporangia germination on leaf surfaces compared with the noninduced control (Yan *et al.,* 2002). Treatment of strawberry plants with the putative elicitor of disease resistance acibenzolar-*S*-methyl, induced resistance against *Phytophthora cactorum,* the causal agent of crown rot (Eikemo *et al.,* 2003). Cohen *et al.,* (1993) demonstrated that jasmonic acid and jasmonic methyl ester (naturally occurring growth regulators) play a role in inducing local and systemic resistance against *P. infestans* within potato and tomato plants. Certain strains of *Pantoea agglomerans* also contributed to systemic acquired resistance in cucumber and *Arabidopsis* (Zhang *et al.,* 1998) and in radish, inducing resistance to bacterial leaf spot (Han *et al.,* 2000).

Designing a set-up suitable for studying the possibility of ISR as a mechanism of inhibition by the potential BCAs in this investigation proved problematic. However, Simons *et al.,* (1996) successfully used method **4.2.9.2** as a gnotobiotic system for studying interactions with tomato plants. This might be for a number of reasons. High levels of contamination were experienced in this investigation possibly because the work was conducted in a microbiology laboratory which would have a reasonable basal level of contaminants. In addition the high levels of ethylene produced by potato required efficient gaseous exchange which was probably not obtained by cotton and foil, which had to be fitted tightly into the tubes to reduce contamination risks. Using Magenta vessels with vented lids was more successful as the problem of ventilation was immediately solved and the set-up was much simpler. The use of sand, however, desiccated the Magenta

atmosphere and eventually the MS medium inhibited plant growth and was therefore unacceptable. The use of layers of medium as a means to ensure separation of the root system and foliage was unsuccessful as the potato plantlet roots did not grow through the layers but along the lower plane of the upper layer. This both retarded plant growth and did not allow any contact with the test bacteria and so was also of no use.

Conn *et al.*, (1997) successfully used a gnotobiotic system to study interactions between potatoes and plant-growth promoting rhizobacteria. The design was simply a test tube with plastic caps which allowed sufficient air exchange, sealed with Micropore surgical tape (3M). Nodal explants were suspended in bacterial suspensions and then placed in the test tubes. This approach is likely to be unsuitable for determining if the bacteria are inducing resistance in the plant because the method of dipping the explant in the bacterial suspension would provide a high possibility of the foliage become contaminated (van Loon *et al.*, 1998).

The results in this chapter only suggest possibilities of the manner in which the potential BCAs may be suppressing disease *in vivo*. In order to determine definitively that a detected mechanism is responsible for disease inhibition observed, a range of methods has been employed by researchers. Most commonly, mutants of the BCA are generated, lacking the characteristic presumed to be the mode of action. If the minus mutant fails to demonstrate any level of disease prevention, it can be concluded that the lacking characteristic is responsible (Handelsman and Stabb, 1996). For example, *Pseudomonas fluorescens* strain Pf-5, which produces many antifungal metabolites including the antibiotic pyrrolnitrin, suppresses *Pyrenophora tritici-repentis in vitro*. A pyrrolnitrin deficient mutant did not produce detectable levels of the antibiotic and did not inhibit the pathogen (Pfender *et al.*, 1993). In the context of antibiotics, over-producing mutants, reporter genes or probes and the isolation and characterisation of genes or gene clusters have been achieved (Whipps, 2000, Raaijmakers *et al.*, 2002). More recently reporter gene systems have been used as markers to monitor populations of introduced strains and to provide information on the transcriptional activity of specific antibiotic biosynthetic genes *in vivo*. This method does not, however, provide accurate information on the amount of antibiotic produced. High performance liquid chromatography (HPLC) has proved one of the most successful techniques, quantitatively detecting

production of phenazine-1-carboxylic acid, herbicolin A, pyrrolnitrin, pyoluteorin, surfactin and 2,4-diacetylphloroglucinol *in situ*. Siderophores have been studied by application of the purified siderophore with synthetic iron chelators (Raaijmakers *et* al., 2002). Some of these approaches would be the next stages in establishing the mode of action of the potential BCAs selected by this investigation.

CHAPTER V

Multiple BCAs, Plant Growth Promotion Potential and Multiple target pathogens

5.1 Introduction

Inoculation of disease conducive soils with a single biocontrol strain rarely leads to the level of suppression observed in naturally disease suppressive soils and the positive effects with single inoculants are often inconsistent (de Boer *et al.*, 1999). This natural suppression is proposed to result from consortia of microorganisms and mechanisms. The application of combinations of BCAs may more closely mimic the natural environment, resulting in a higher level of protection, with reduced variability and have the potential to suppress multiple plant diseases (Guetsky *et al.,* 2001, de Boer *et al.*, 2003). For example, the application of two strains of *Pseudomonas putida* resulted in enhanced disease control of Fusarium wilt of radish. When one strain failed to suppress disease in the single application, the combination still resulted in disease control (de Boer *et al.*, 2003). Combinations of fluorescent pseudomonads improved biocontrol of the disease, take-all and growth of wheat illustrating the importance of additive and interactive effects among introduced BCAs (Pierson and Weller, 1994). It is also possible that the level of disease control achieved by mixtures of effective BCAs may be less influenced by factors such as crop cultivar than applications of a single BCA (Fukui *et al.*, 1999b).

The observation that certain rhizobacteria stimulate plant growth is well documented. Kloepper and Schroth (1978) first described this phenomenon as 'plant growth-promoting rhizobacteria' (PGPR), a term which is now widely used. The properties of PGPR offer great promise for agronomic application (Persello-

Cartieaux *et al.,* 2003). Large-scale application of PGPR to crops could reduce the use of chemical fertilisers and increase crop yield (Bloemberg and Lugtenberg, 2001). In 1985, Rhizobia became the first commercial PGPR, marketed as 'nitragin'; now similar products are well established as agricultural products in the USA, Europe, Australia and India (Arshad and Frankenberger, 1991).

PGPR may increase plant growth directly by associative N_2 fixation, solubilizing nutrients such as phosphate, promoting mycorrhizal function, regulating ethylene production in roots, releasing phytohormones, including auxins and cytokinins, and by decreasing heavy metal toxicity (Whipps, 2001). Synthesis of siderophores, which can provide iron to the plants, and of enzymes that can modulate plant growth and development may also contribute to growth promotion. A particular bacterium may promote plant growth and development using any one, or more, of these mechanisms. Moreover, a bacterium may utilise different traits at various times during the life cycle of the plant (Penrose and Glick, 2003).

The ability of soil and rhizosphere microorganisms to produce plant growth regulators such as auxins and cytokinins is widespread. Auxins can have a range of effects on plant development, the critical characteristic being their ability to induce cell elongation in stems. The previous chapter demonstrated isolates 6, 9, 10 and 13 (all *Enterobacter* spp.) from this investigation to be positive for production of the ubiquitous auxin, indole-3-acetic acid (IAA). Production of this plant hormone by rhizobacteria has been demonstrated to contribute to plant growth promotion (Xie *et al.,* 1996). Cytokinins are N^6 substituted aminopurines that act as plant growth promoters and influence physiological and developmental processes of plants such as cell division, seed germination, root development, accumulation of chlorophyll, leaf expansion and delay of senescence (Garcia de Salamone *et al.,* 2001). Rhizobacteria secreted gibberellins and regulation of ethylene production can also contribute to plant growth promoting mechanisms.

Some BCAs are effective against more than one phytopathogen. The use of mixtures of PGPR successfully controlled multiple cucumber pathogens (Raupach and Kloepper, 1998). This broad spectrum activity holds practical and economical advantages. *P. infestans* is responsible for extensive losses of

tomato crops across Europe and North America annually (Deahl *et al.*, 1996). BCAs that could prevent both potato and tomato against late blight would have widespread application and value.

5.2 Materials and Methods

5.2.1 Determination of a consortium of three BCAs

The same tuber bioassay used to select the suppressive soils and subsequently 15 suppressive bacterial isolates (Fig. 2.1) was employed to determine a consortium. Dilution series of each isolate were tested (1:1) against *P. infestans.* Isolates and tubers were prepared as in section **2.2.4**. Isolate 1 was tested at approximate concentrations of 1 x 10^8 cfu/ml, 5 x 10^7 cfu/ml, 2.5 x 10^7 cfu/ml, 1 x 10^7 cfu/ml and 1 x 10^6 cfu/ml. Isolates 2 to 15 were tested at the first three concentrations only.

The three isolates that exhibited the highest levels of suppression proportional to cfu/ml were selected to be tested as a combination. The combination was tested at the same concentrations as isolate 1.

5.2.2 Plant growth promotion of tomato seedlings

5.2.2.1 Promotion of shoot and foliage development

An assay used by Silva *et al.*, (2003) as a root colonisation bioassay was adapted. Tomato seeds (15) were sterilised in 2% NaOCl, rinsed thoroughly with sterile water and soaked overnight in 1.5ml of antagonist suspension, prepared as in section **2.2.4**. Seeds were then transferred to Magenta vessels containing 25ml 1% water agar and incubated in a controlled environmental chamber at 22°C with a 12.5/11.5 day/night cycle for 6 weeks during which measurements of shoot development were taken. Foliage and root development was also recorded. The experiment was repeated once.

5.2.2.2 Promotion of root development

The same assay was utilised to study root development, except the tomato seeds were transferred into Petri dishes containing 15ml 1% agar. Plates were also observed for any evidence of root colonisation.

5.2.3 Potato root development and colonisation by antagonist

The developing root systems of potato explants in experiment **4.2.9.4** were observed for any antagonism influence and colonisation.

5.2.4 Testing of antagonists against tomato late blight

The tomato blight strain of *P. infestans* was isolated from tomato cv. Gardeners Delight (obtained courtesy of Louise Cooke of The Queen's University of Belfast). It is metalaxyl sensitive and A1 mating type. To ensure compatibility between the *P. infestans* strain and the tomato cultivar, cv. Gardener's Delight (Thompson & Morgan) was used for all experiments.

5.2.4.1 Treated tomato seedlings maintained in Petri dishes

Tomato seeds (15 per treatment) were soaked overnight in 1ml solution of each bacterial isolate. The antagonists were suspended in 10mM potassium phosphate buffer (pH 7.2) and adjusted to an absorbance of 0.4 at 620nm (corresponding to approximately 2.35 x 10^9 cfu/ml). The control was seeds soaked in phosphate buffer only. The seeds were transferred to a *P. infestans* suspension (1ml) for approximately five hours. The inoculated seeds were transferred to a Petri dish containing sterile filter paper and 1ml sterile distilled water. The Petri dishes were sealed, wrapped in silver foil and incubated at 18 to 20°C. After the seeds had germinated (four days) the foil was removed and the plates subjected to a day/night light cycle of 14/10 h. After four weeks the seedlings were weighed. This experiment was repeated as above except the seeds were soaked in the bacterial solutions and placed in Petri dishes without inoculation with *P. infestans.* Each Petri dish was opened 2 weeks later and sprayed with 2ml sporangial suspension of *P. infestans.* The plates were resealed and incubated for a further two weeks in the same conditions, after which the seedlings were weighed.

5.2.4.2 Treated tomato seedlings maintained in soil

Tomato plants were grown with various seed and soil treatments, see Table 5.1.

Table 5.1 Soil, seed and subsequent treatments to test effectiveness of antagonists against tomato late blight

Treatment number	Seed treatment	Soil treatment	Subsequent treatment
1	None	20ml antagonist suspension	After 1 month sprayed with 1ml *P. infestans* suspension
2	Soaked overnight in 2ml antagonist suspension	4ml *P. infestans* suspension	None
3	Soaked overnight in 2ml *P. infestans* suspension	20ml antagonist suspension	None

Antagonist suspensions were prepared as in section **2.2.4**. Plant pots containing approximately 250g compost were prepared. In all cases the control was the same as the antagonist treatment except phosphate buffer only was used. Seeds (10) were planted in the soil and grown for one month at 18 to 20 °C with day/night light cycle of 14/10 h. The pots were kept in trays covered with transparent plastic sheets. The pots were watered regularly with distilled water. Plants were examined for disease incidence at 6 weeks.

All assays failed to induce tomato late blight incidence in the control or in any treatments and were therefore of no use.

5.3 RESULTS

5.3.1 Determination of a consortium of two or three BCA

All 15 isolates were tested at a concentration of 100 cfu/nL, 50 cfu/nL and 25 cfu/nL (Fig. 5.1 to 5.15). Soft rot was evident in many of the treatments and accounted for much of the variation observed. However, disease levels at all three concentrations for all 15 isolates were still lower than observed in the controls.

Isolate 1 exhibited 17% disease compared to the control (100%) of *P. infestans* only inoculated into a tuber. Disease suppression decreased in a clear linear trend with concentration (Fig. 5.1). Variation was not as high as observed in a number of other treatments. A number of the isolates demonstrated similar patterns of suppression to isolate 1 but with higher levels of variation (Fig. 5.2, 5.3, 5.6, 5.7, 5.8 and 5.9). Isolate 3 suppressed disease efficiently at 100 cfu/nL, this decreased to approximately 30% at 50 cfu/nL with increased variation. Disease suppression did not however decrease further when the antagonist concentration decreased to 25 cfu/nL (Fig. 5.13). Suppression by isolate 5 was less efficient with higher variation than a number of other isolates (Fig. 5.4). Isolates 6 and 12 exhibited high levels of suppression at 100 cfu/nL and 25 cfu/nL but surprisingly lower levels at 50 cfu/nL (Fig. 5.5 and 5.10). Isolates 7 and 14 demonstrated high levels of suppression with low variation at 100 cfu/nL and 50 cfu/nL. Suppression decreased and variation increased at 25 cfu/nL (Fig. 5.14 and 5.15). Disease suppression by isolate 13 was less consistent, with relatively high numbers of disease lesions observed with the highest concentration of antagonist (100 cfu/nL, Fig. 5.11). Isolate 15 exhibited reasonable disease suppression (approximately 35% of the control) but high levels of variation were evident (Fig. 5.12).

From these results, isolates 3, 7 and 14 were selected to test in combination.

FIG. 5.1 to 5.15. The control treatment was *P. infestans* inoculated with sterile phosphate buffer only. Points are means of three replications with standard error bars.

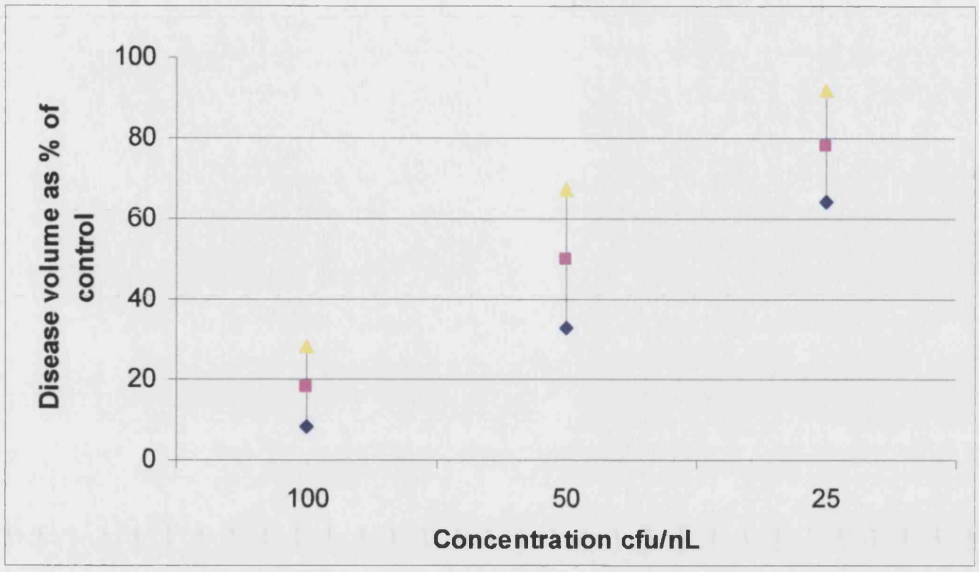

FIG. 5.1 Isolate 1 bioassayed against *P. infestans*.

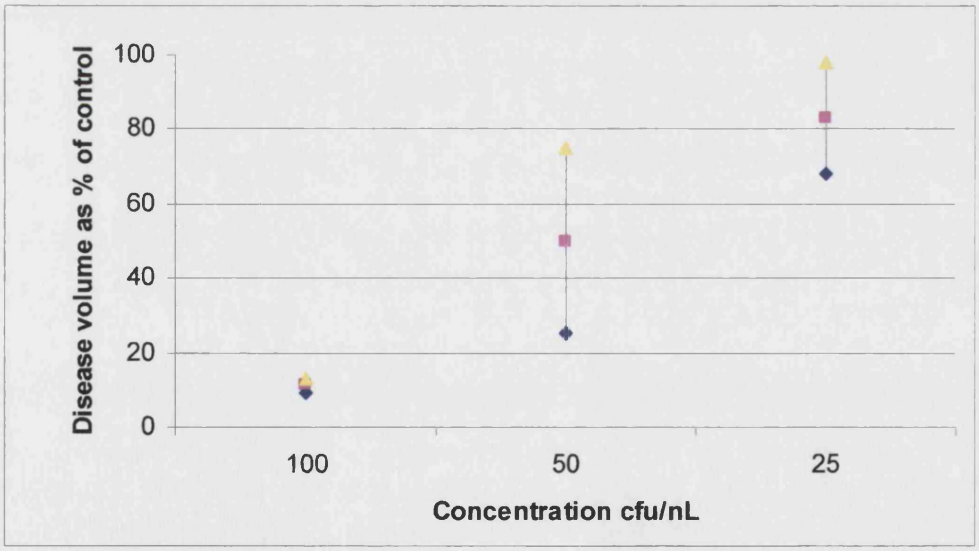

FIG. 5.2 Isolate 2 bioassayed against *P. infestans*.

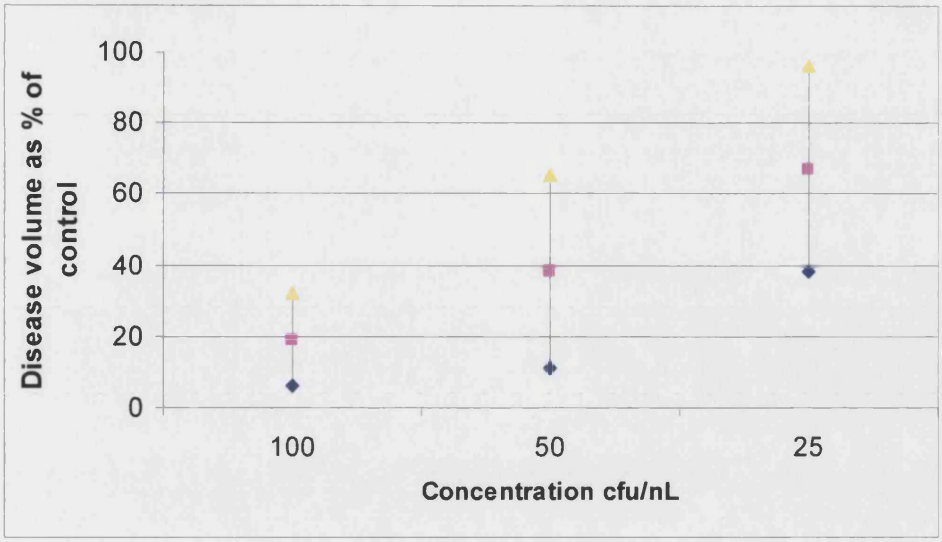

FIG. 5.3 Isolate 4 bioassayed against *P. infestans.*

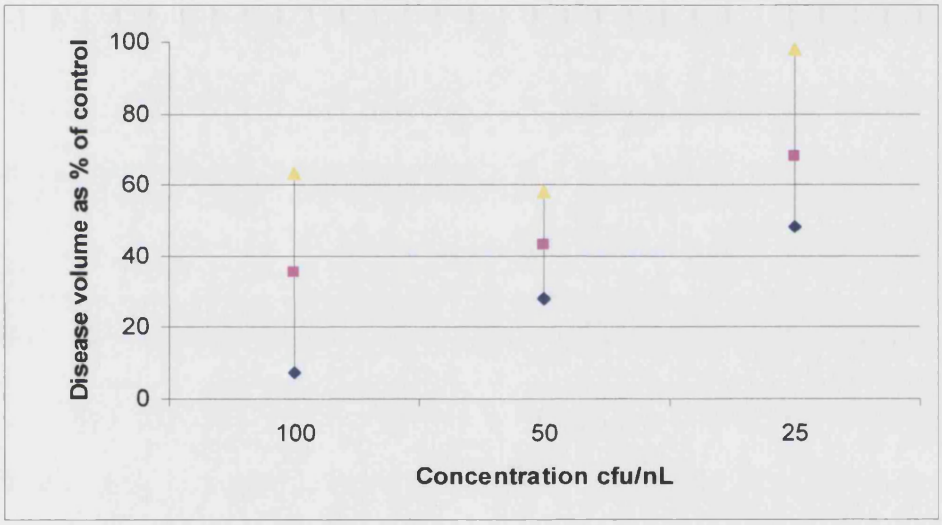

FIG. 5.4 Isolate 5 bioassayed against *P. infestans.*

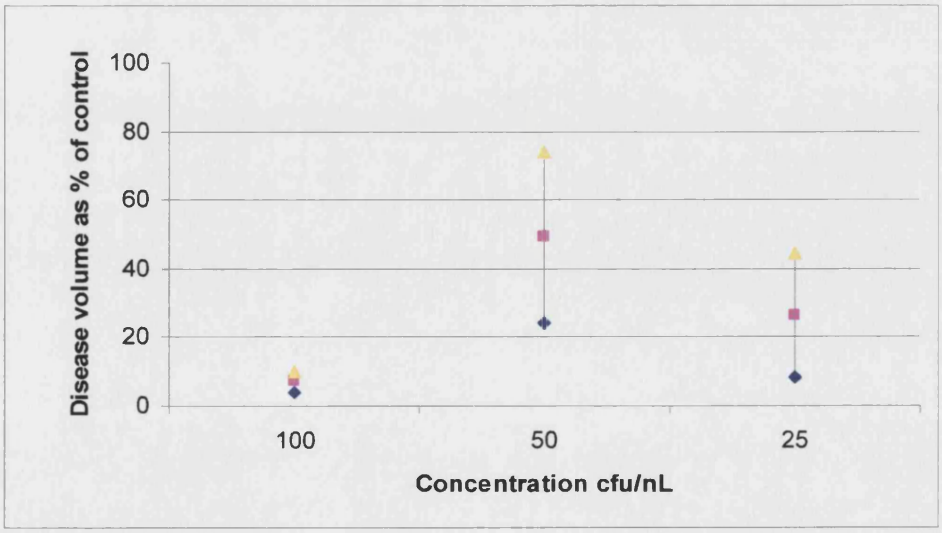

FIG. 5.5 Isolate 6 bioassayed against *P. infestans.*

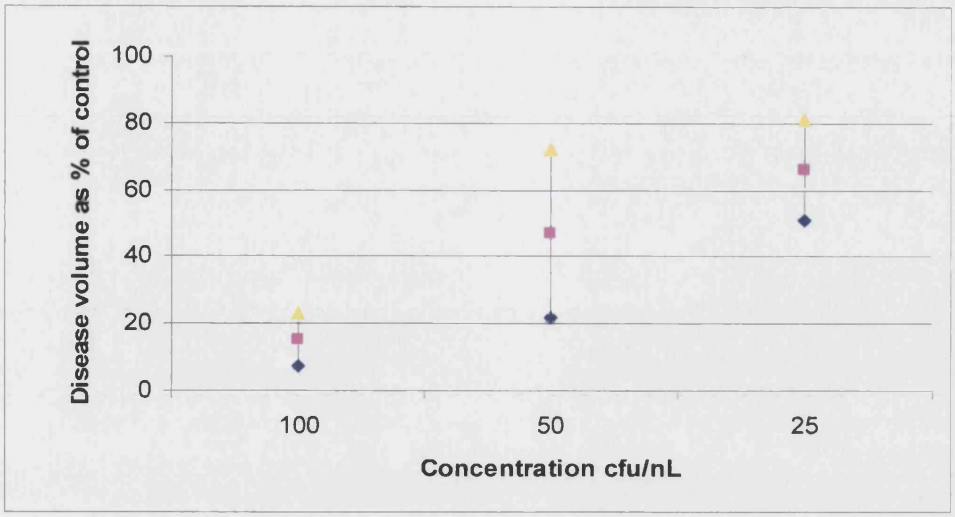

FIG. 5.6 Isolate 8 bioassayed against *P. infestans*.

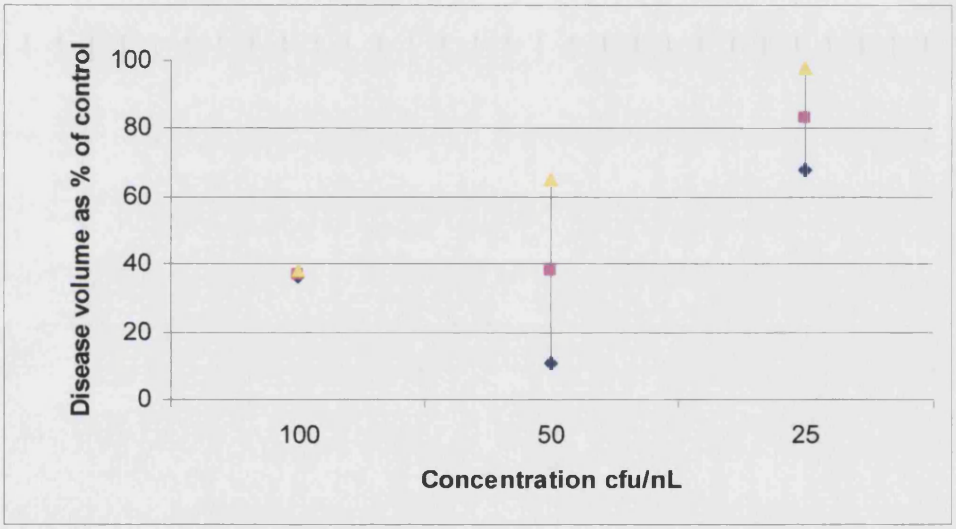

FIG. 5.7 Isolate 9 bioassayed against *P. infestans*.

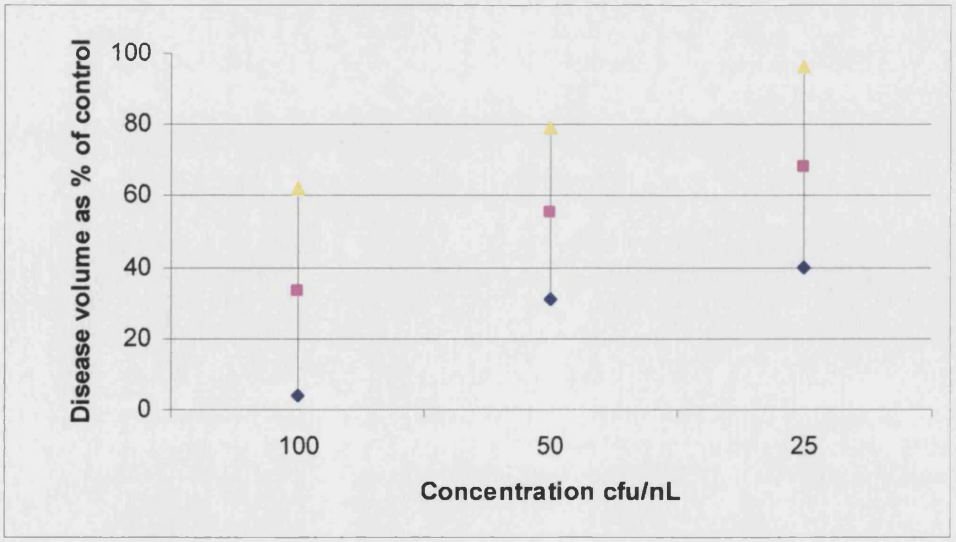

FIG. 5.8 Isolate 10 bioassayed against *P. infestans*.

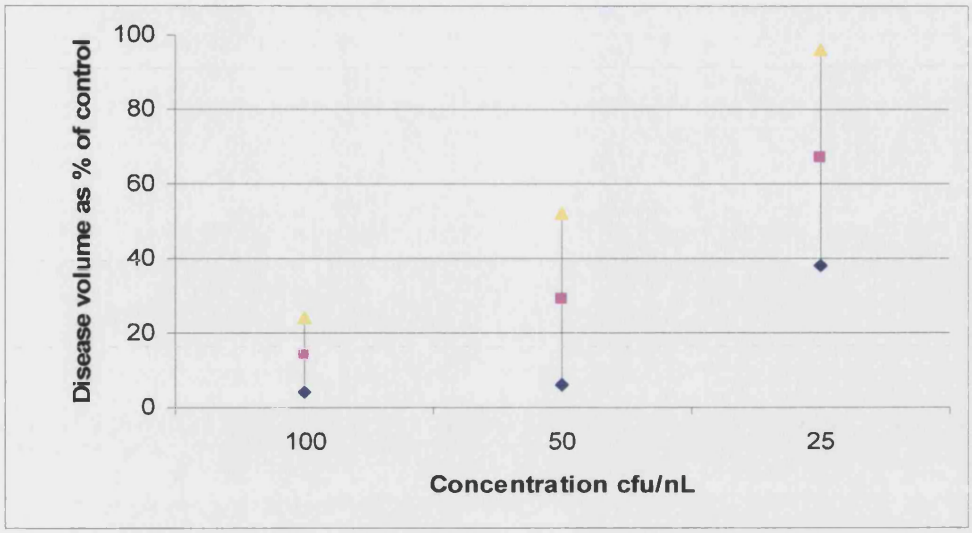

FIG. 5.9 Isolate 11 bioassayed against *P. infestans*.

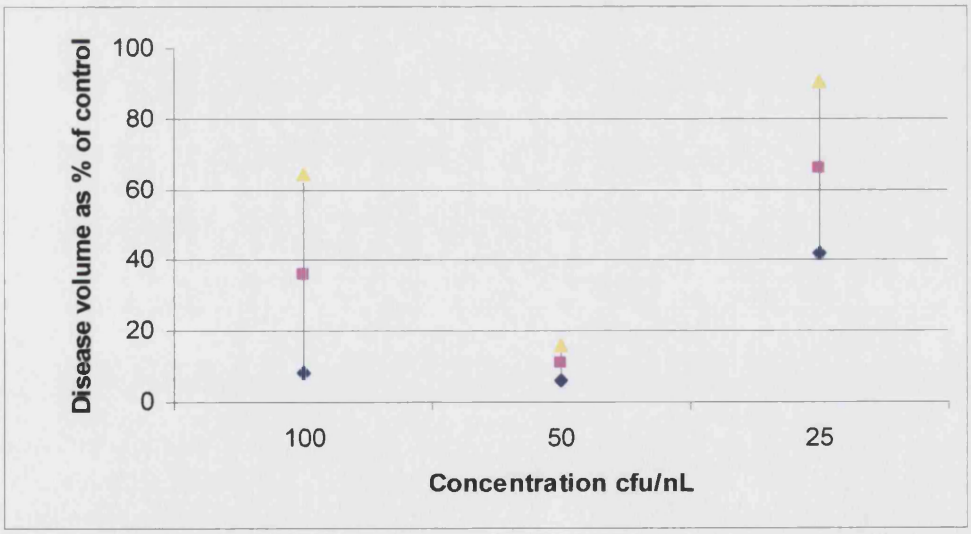

FIG. 5.10 Isolate 12 bioassayed against *P. infestans*.

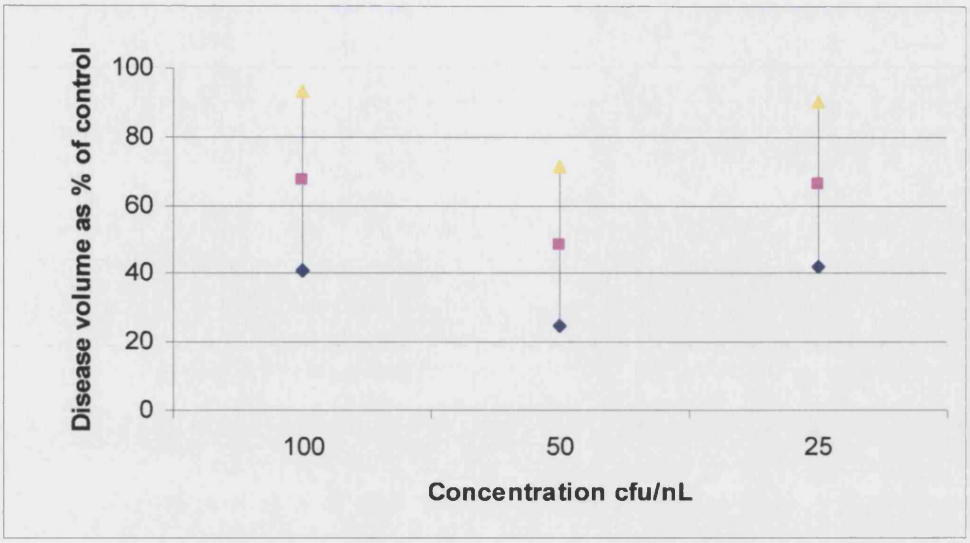

FIG. 5.11 Isolate 13 bioassayed against *P. infestans*.

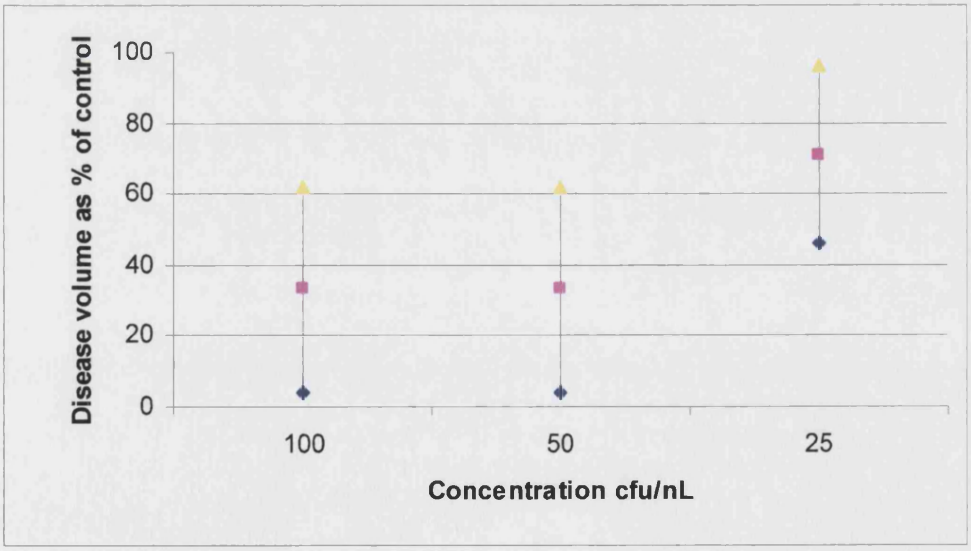

FIG. 5.12 Isolate 15 bioassayed against *P. infestans*.

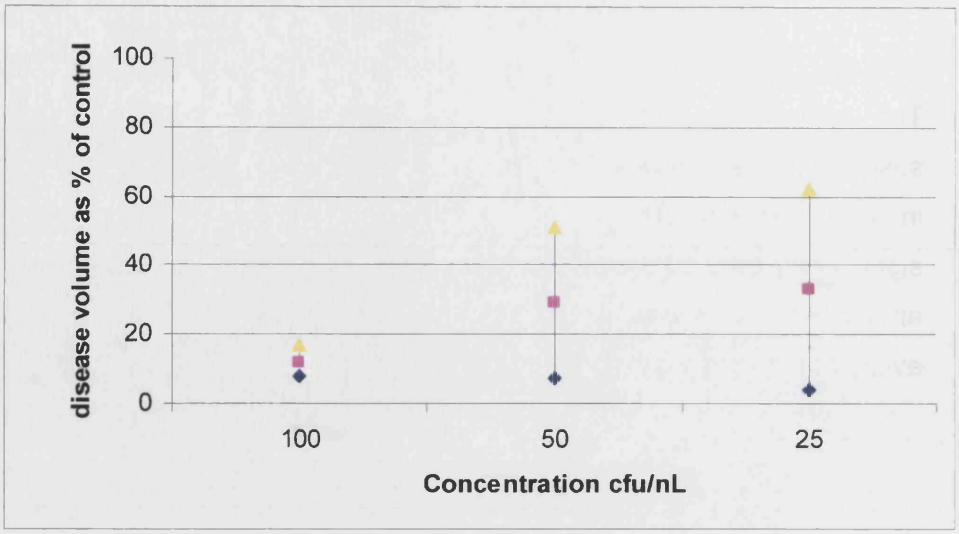

FIG. 5.13 Isolate 3 bioassayed against *P. infestans*.

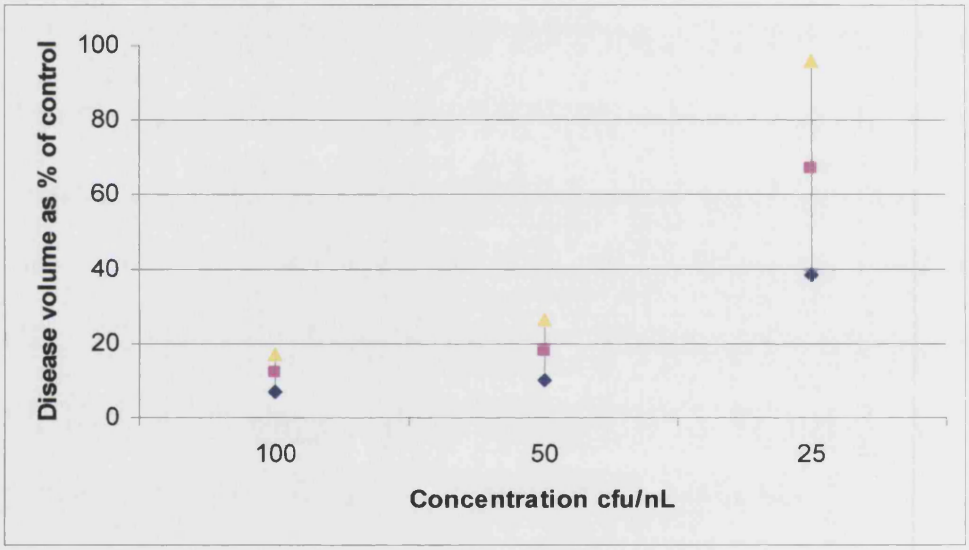

FIG. 5.14 Isolate 7 bioassayed against *P. infestans*.

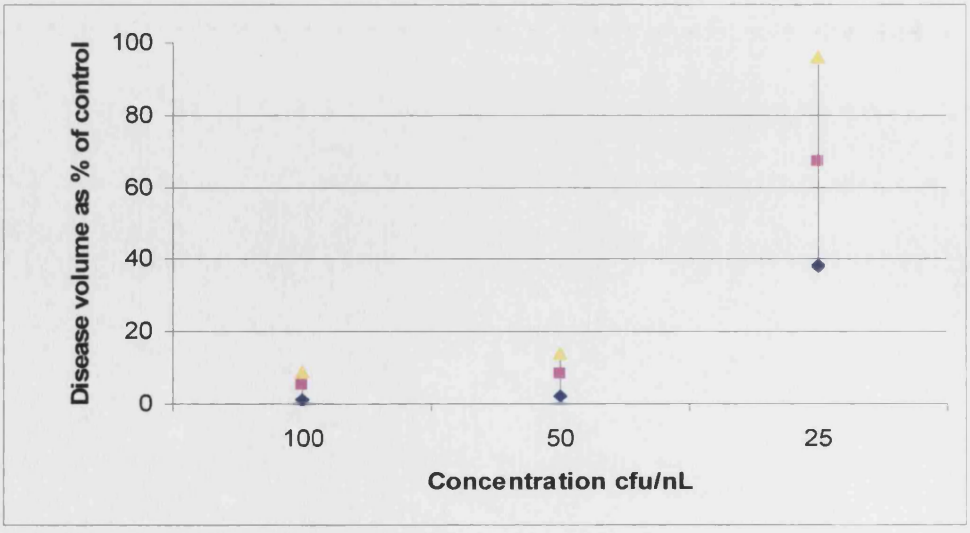

FIG. 5.15 Isolate 14 bioassayed against *P. infestans*.

The total number of antagonist propagules in the combination inoculation suspension was the same as the total amount of propagules in the assays of individual antagonists. The combination of isolate 3, 7 and 14 resulted in significantly (p=0.001) higher levels of consistent disease suppression at lower antagonist concentrations (25 cfu/nL). Reduced disease suppression was still evident at 10 and 1 cfu/nL with increased variability (Fig. 5.16).

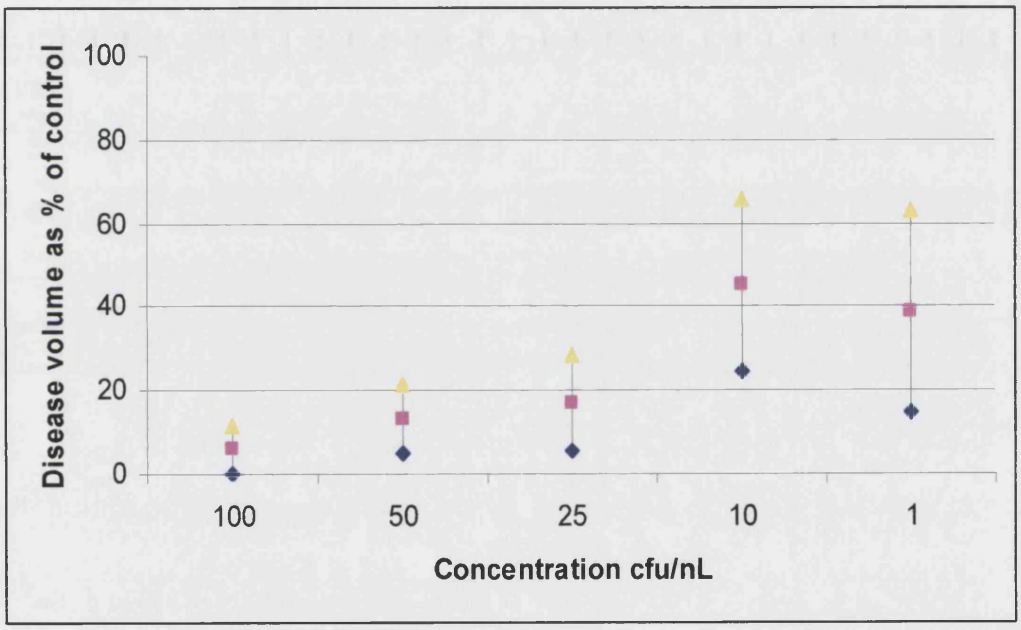

FIG. 5.16 Consortium of isolates 3, 7 and 14 bioassayed against *P. infestans*.

5.3.2 Plant growth promotion of tomato seedlings

5.3.2.1 Promotion of shoot and foliage development

Treatment of seeds with isolates 1, 3, 4, 6, 7, 8, 9, 10, 11, 12 and 14 resulted in significant stem elongation (p=0.001) (Fig. 5.17 and 5.18).

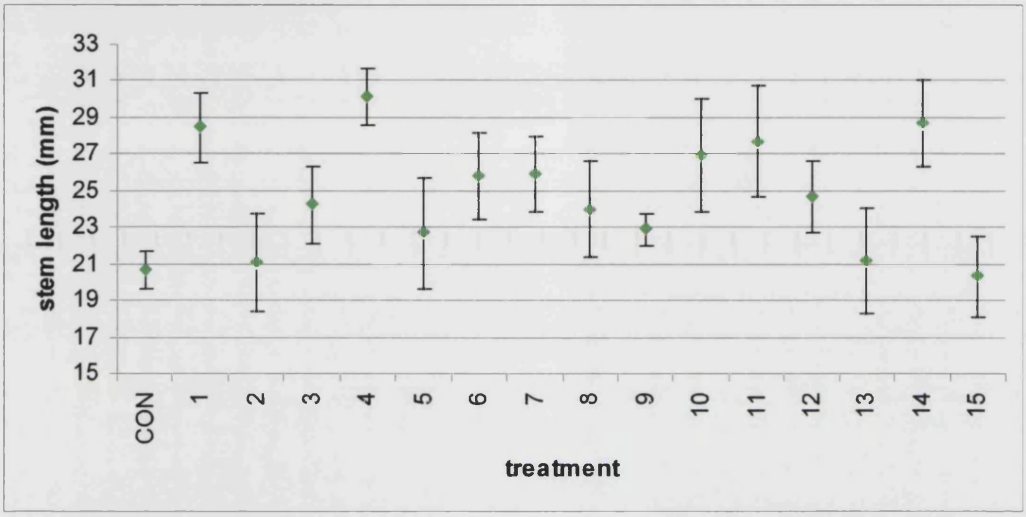

FIG. 5.17 Stem length of tomato seedlings treated with potential BCAs after 26 days, the control was seeds soaked in phosphate buffer only. Points are the mean of 20 seedlings with standard error bars.

FIG. 5.18 Stem elongation of tomato seedlings treated with isolate 1 (seedlings on right). The control was tomato seeds treated with phosphate buffer (seedlings on left).

The same trend was evident at 41 days. The foliage was observed at 33 days and no treatment was significantly different from the control except isolate 8 and 14. The control had an average of 2.78 leaves per seedling compared to 5.2 per seedling treated with isolate 8 and 4.95 per seedling treated with isolate 14 (Fig. 5.19).

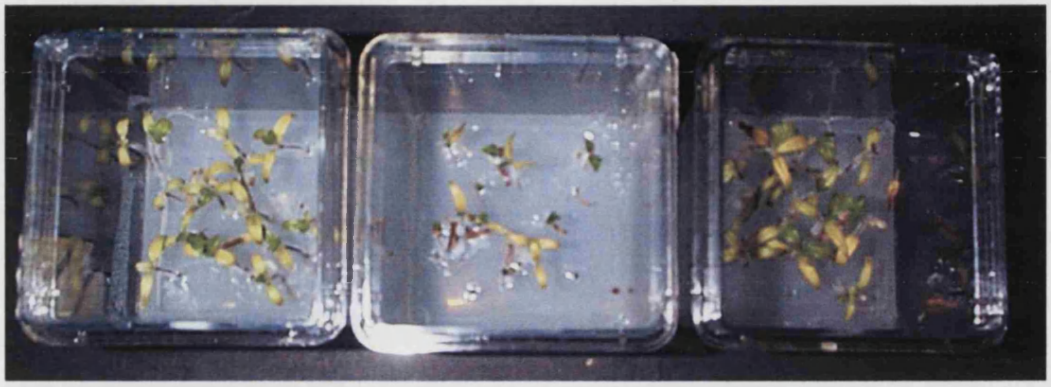

FIG. 5.19 Tomato seedlings treated with isolate 14 (left) and isolate 8 (right) exhibiting advanced foliage development compared to the control of tomato seedlings treated with phosphate buffer (centre). Each Magenta vessel contains 15 tomato seedlings.

5.2.2.2 Promotion of tomato seedling root development

Substantial root elongation was evident in tomato seedlings treated with isolates 2, 4, 5, 6, 9, 11, 13, 14, 15 and the consortium of isolates 3, 7 and 14 (Fig. 5.20).

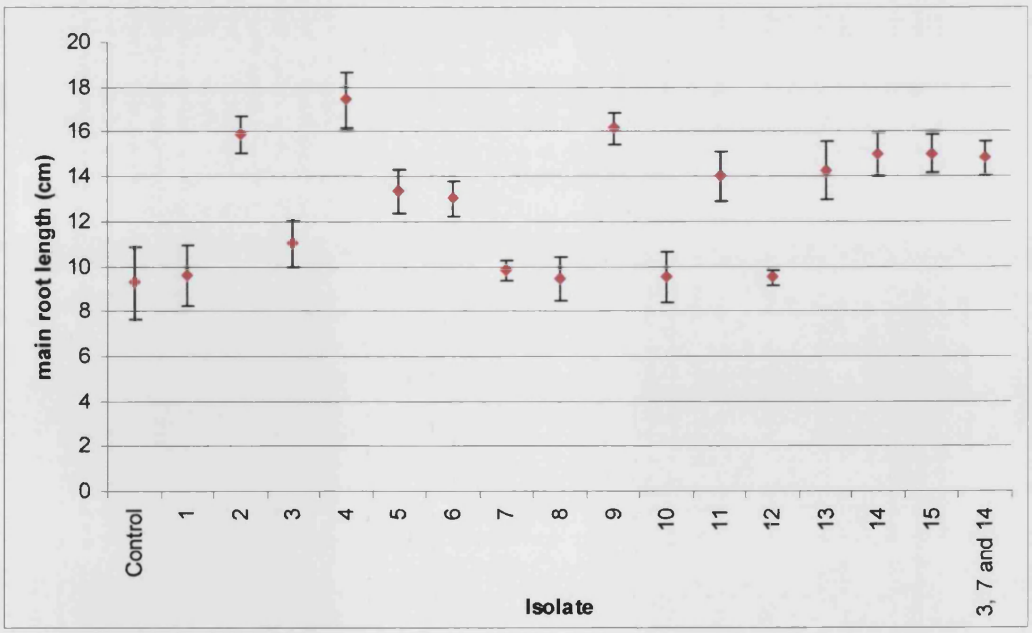

FIG. 5.20 Main root length of tomato seedlings treated with potential BCAs after 26 days. Control treatment was tomato seedlings soaked in phosphate buffer.

5.3.3 Potato root development and colonisation by antagonist

Isolate 1 was visibly colonising the explant roots (Fig. 5.21).

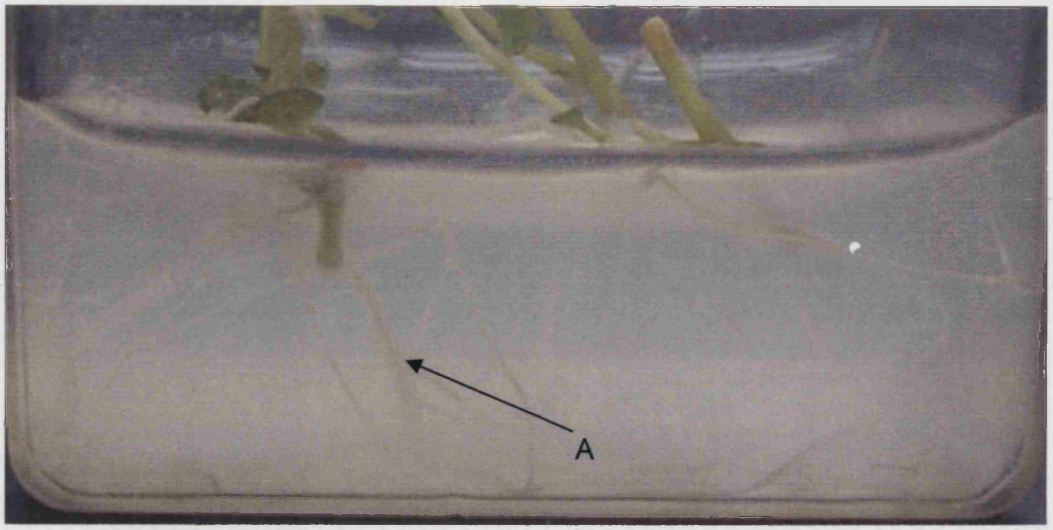

FIG. 5.21 Isolate 1 colonising potato explant roots (A).

Isolate 6 was completely inhibiting explant root growth. Shoot development was still evident, however almost no root development had occurred (Fig. 5.22).

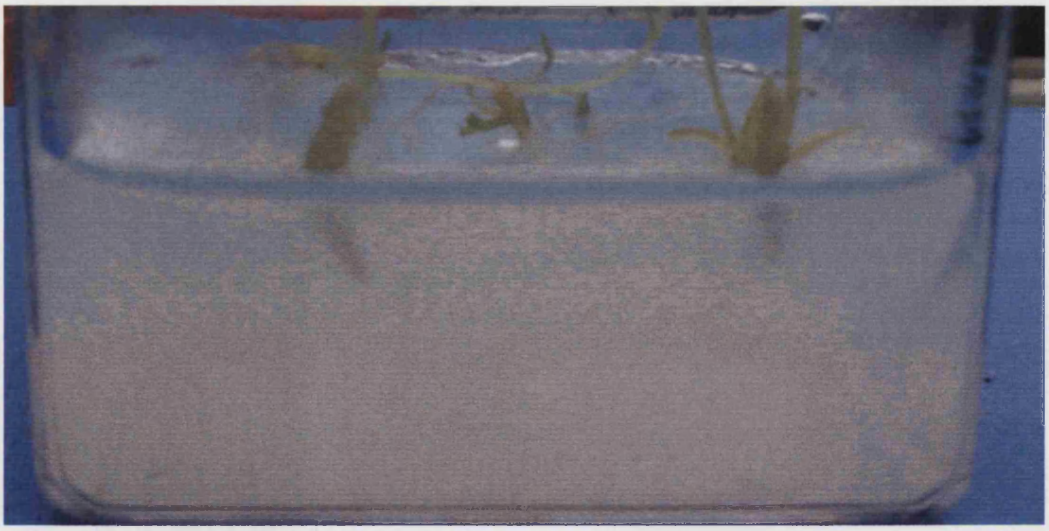

FIG. 5.22 Isolate 6 inhibiting potato explant root development.

High levels of contamination were experienced with this experiment. It was therefore not possible to study the effect of the other isolates on potato explant development.

5.4 Discussion

In order to determine which isolates were the best candidates to select for a potential control consortium, all isolates were tested in dilutions. All isolate concentrations suppressed disease more than the control. However, only isolates 3, 7 and 14 exhibited high levels of suppression at decreasing propagule concentrations. In addition these three isolates individually significantly promoted tomato seedling stem elongation (Fig. 5.17). Isolates 3 and 7 did not promote root development individually although significant main root elongation was evident by isolate 14 (Fig. 5.20) and this was still evident in the roots of seedlings treated with all three isolates (Fig. 5.20). Isolate 7 produced an antibiotic compound that caused the hyphae of *P. infestans* to kink, inhibiting mycelial growth (Fig. 4.22), while isolate 3 produced very high levels of siderophores (Fig. 4.30). Isolate 14, which was the most inhibitory towards *P. infestans* in the bioassay (Fig. 5.15), produced no siderophores (Fig. 4.30) and did not appear to be highly toxic in antibiotic assays (**4.2.1.1** and **4.2.1.2**). Higher levels of consistent control were achieved with lower antagonist populations when the consortium was applied. The results (Fig. 5.16) suggest additive control. Further experiments would be required to determine more precisely the levels of additive or synergistic activity, if any, between the three isolates.

Large numbers of researchers cite the benefits to biocontrol that may be achieved by the using of multiple BCAs, particularly if they exhibit different or complementary modes of action or abilities to colonise root microsites (Whipps, 2001, Guetsky *et al.,* 2002). The probable different modes of action of isolates 3, 7 and 14, if applied in combination, would avoid placing a single selection pressure on the *P. infestans* population. Additionally these different mechanisms may be more effective in different conditions, which would contribute to decreasing the variability of the biocontrol. Schisler *et al.,* (1997) successfully selected a pair of BCAs antagonistic to Fusarium dry rot of potatoes which reduced disease to approximately 70% versus controls, a level of control comparable to that obtained with 100 times the inoculum dose of a single antagonist strain.

The approach used in this investigation for selecting a consortium has limitations because synergism between isolates may be missed. However the alternative method of testing each pair combination would involve a scale of experiment

which was not feasible in this investigation. A number of alternative methods has been used to develop effective combinations of BCAs. For example, several indigenous bacterial strains in guttation fluids suppress *Xanthomonas campestris*, the causal agent of bacterial blight of anthurium. Inhibition of growth was not observed in filter-sterilised guttation fluids but was restored to original levels only by reintroducing a specific mixture of five bacteria (Fukui *et al.*, 1999a). Further research involving these bacteria suggested the most appropriate method for determining a consortium of BCAs is to select an effective mixture first and then remove ineffective strains (Fukui *et al.*, 1999b). Janisiewicz (1996) successfully used another method to determine a combination of two antagonists which were consistently superior in their control of blue mold on apples to each individual strain. This approach used nutritional clusters based on utilisation of 95 carbon sources in Biolog plates. The most promising antagonists from different clusters were paired in subsequent tests, with isolation site also taken into account. Nutritional profiles of each isolate from the antagonistic pair revealed significant differences in carbon catabolism. These differences caused niche differentiation and allowed populations of both antagonists to flourish in the same wound.

Determining the relationship between antagonist and pathogen propagules, in this case by bioassaying a dilution series of the antagonist, is one of the first steps in determining dose-response relationships. Such information is extremely important for ensuring reliable biocontrol strategies. Commercially, the increase in production costs associated with developing more than one BCA must be proportional to the increase in disease control. This investigation therefore selected three microorganisms for further development.

Classical selection of BCAs has focused on a single target pathogen and has not accounted for non-target pathogens causing diseases on the host plants (Raupach and Kloepper, 1998). This has been evident in this investigation with bacterial soft rot of potatoes providing one of the biggest limitations to the selection procedure employed. The development of a consortium which also suppressed this disease would have huge advantages. It is possible that the lower levels of variation in suppression exhibited by some isolates may be attributed to an ability to suppress soft rot (for example, isolates 1, 7 and 14). In some cases, for example isolate 6, a lower concentration of antagonist resulted in a higher level of disease suppression. This is probably as a result of the small sample number and the occurrence of soft rot lesions on tubers treated with the

higher antagonist concentrations. This is consistent with the high levels of variation observed for the higher concentration (Fig. 5.6).

In order to determine the consortium of BCAs all 15 were retested at the same concentration against the same pathogen level as had been used to initially select antagonists in order to allow confirmation of strain activity. Isolates were stored in liquid nitrogen (Chapter III) throughout the investigation to minimise possible phenotypic mutations, however alterations or contamination may still be possible. The levels of suppression observed (Fig. 5.1 to 5.15) confirm that the phenotype, in terms of disease suppression, had not changed through the course of the investigation.

Plant growth promotion experiments were conducted primarily using tomato seedlings. This was owing to the greater simplicity of using tomato seeds compared to potato explants. Other researchers have, however, successfully studied plant growth promotion of potatoes. Frommel *et al.,* (1993) demonstrated growth promotion of potato by a non-fluorescent pseudomonad by using inoculated suberised seed tuber pieces maintained in a greenhouse. Increased root number, root dry weight, haulm dry weight and stem length of potato plantlets were observed. Bacterisation also increased leaf hair formation, secondary root branching and total plant lignin content (Frommel *et al.*, 1991). This approach was unsuitable for this investigation because of space constraints.

Stem elongation was observed by a number of the potential BCAs selected in this investigation. Plant growth promotion and biocontrol by the same strains of PGPR has been observed by other researchers (Yan *et al.,* 2000). A number of *Enterobacter* species (isolates 4, 5, 6, 7 and 8) exhibited substantial stem elongation. Members of this genus, as well as *Pseudomonas* and *Klebsiella,* are free-living nitrogen-fixing bacteria maintaining an interaction with host plants which results in root associated nitrogen-fixing activity and may promote plant development (Maunuksela, 2001). Such bacteria, characterised by their ability to transform atmospheric nitrogen into ammonia that can be used by plants, are known as diazotrophs (Dobbelaere *et al.,* 2003). Most research concerning the contribution of biological nitrogen fixation to plant growth has, however, failed to establish a strong link indicating that associative diazotrophs exert their positive effects on plant growth directly or indirectly through a combination of mechanisms (Dobbelaere *et al,* 2003).

The stem elongation observed in this investigation is probably largely attributable to GA_1, the most active gibberellin in plants and the first to be discovered (Dobbelaere *et al.*, 2003). PGPR *Bacillus pumilis* and *B. licheniformis* have been shown to produce high amounts of physiologically active gibberellins, inducing stem elongation on alder plants (Gutiérrez-Mañero *et al.*, 2001). The researchers also observed promotion of leaf growth area, as observed with isolate 8 and 14 in this investigation, but from the data could not conclude whether this was due to only gibberellins and/or auxins or even additional plant growth promotion substances present in the medium.

Plant growth promotion may also be as a result of bacterial regulation of the production of ethylene in developing seedlings. Interestingly analysis of PGPR by an IAA producing *P. putida* strain demonstrated that plants exposed to the bacterial auxin produced higher levels of 1-aminocyclopropane-1-carboxylate (ACC) synthase, an enzyme normally used by plants to produce ethylene. Exuded ACC is hydrolysed by an ACC deaminase, a bacterial enzyme present in *Enterobacter cloacae* and several *Pseudomonas* strains. The uptake and subsequent hydrolysis of ACC by the PGPR decreases the amount of ACC outside the plant which must exude increasing levels of ACC to maintain the equilibrium between internal and external ACC levels. The bacterium uses the ACC as a source of nitrogen and the plant shows better root elongation as its internal level of ethylene decreases (Persello-Cartieaux *et al.*, 2003). Penrose and Glick (2003) recently designed a method for selecting PGPR with ACC deaminase activity, with strains selected by this method having been successfully applied to promote the growth of rice seedlings and soybean plants in the field in China.

Ethylene synthesis in tomato is controlled by the highly transcriptionally regulated genes encoding ACC synthase and ACC oxidase (Klee, 2002) and treatment of potato tubers with IAA has been demonstrated to increase mRNA levels of a cDNA clone encoding an ACC oxidase (Zanetti *et al.*, 2002). It is therefore possible that bacterial IAA production could decrease internal ethylene levels within these plants contributing to growth promotion.

The production of siderophores by rhizobacteria may also promote plant development as this would increase mobility of iron in the rhizosphere, making it

more available to plants and therefore indirectly promoting growth. The exact mechanisms are not fully understood, however, with more efficient plant uptake of synthetic iron chelators or phytosiderophores than of bacterial siderophores (Dobbelaere *et al.,* 2003). A number of the isolates that promoted stem elongation were siderophore producers. Additionally the 1% water agar in which the PGPR experiments were conducted would be sufficiently low in iron to induce siderophore production.

While IAA is associated with cell elongation, division and differentiation there was no direct correlation between isolates that produced IAA (isolates 5, 7, 8, and 9) in this investigation and growth of the seedlings. Isolates 5, 8 and 9 promoted seedling growth less than almost every other strain in the investigation (Fig. 5.17). In contrast, auxin production by rhizobacteria, expressed as IAA equivalents, has been correlated with up to 56.5% increased plant height of *Brassica juncea* seedlings (Asghar *et al.,* 2002). L-tryptophan (L-TRP) is a precursor of IAA and the presence of this compound increases IAA production (Arshad and Frankenberger, 1991). It is possible that no L-TRP or low levels in the medium used in this investigation resulted in low IAA activity and therefore low impact on plant development. However, significant root elongation was evident by isolate 5 and particularly by isolate 9 (Fig. 5.20). The application of pure IAA to wheat increased root length, number of lateral roots and number of root hairs (Asghar *et al.*, 2002) suggesting that root elongation evident in this investigation may be attributable, in some cases, to IAA production. IAA produced by *P. putida* (strain GR12-2) has also been demonstrated to play a major role in the development of the Mung bean (*Vigna radiata)* root systems (Patten and Glick, 2002). Furthermore, high levels of auxin relative to cytokinin are known to stimulate the formation of roots, whereas high levels of cytokinin relative to auxin leads to the formation of shoots (Taiz and Zeiger, 1991). However, 8 isolates exhibiting significant tomato seedling root elongation were not positive for IAA production. This suggests that these isolates may be producing alternative forms of auxin or are stimulating root elongation by another mechanism.

The root inhibition of potato plantlets by isolate 6 may be due to high ethylene or methyl jasmonate levels (Patten and Glick, 2002). Methyl jasmonate, like ethylene, is a naturally occurring growth regulator and signalling molecule, and

has been observed to inhibit root development in *Arabidopsis* (Staswick *et al.*, 1992).

The production of phytohormones by PGPR and the interacting effects on plants are complex, illustrated by the fact that each hormone has been found to be able to affect nearly every phase of plant growth and development. For example, the balance between auxin and cytokinin levels controls cellular differentiation and organogenesis in tissue and organ culture, ranging from shoot proliferation to root formation as the ratio of auxin/cytokinin increases. In addition, auxins and cytokinins stimulate ethylene production synergistically (Dobbelaere *et al.*, 2003). PGPRs may also stimulate plant growth by the promoting uptake of minerals N, P and K and microelements. The use of mutant minus or overproducing PGPR and/or plants are helping to elucidate exact roles.

Isolate 1 is known to produce the compound hydrogen cyanide as discussed in Chapter III and IV. Cyanide may inhibit pathogens but has also been reported as inhibiting plant growth on occasions which might offset the advantages provided by a BCA (Kremer and Souissi, 2001). The substantial stem elongation observed by seedlings treated by isolate 1 indicates that, certainly in terms of tomato, cyanide inhibition is not a problem. This may not be true for potato, with some PGPR promoting potato growth primarily by suppressing cyanide-producing deleterious rhizosphere microorganisms (Weller, 1988).

Plant growth promoting activities by rhizobacteria can be very valuable. An example of large scale application of a PGPR is *Alcaligenes faecalis* (strain A1501), which was isolated from the rice rhizosphere and is now used to improve plant growth of several important crops in China. This strain is able to grow under high salt conditions, maintaining its IAA production and N_2 fixing abilities. Dinitrogen fixation by A1501 resulted in an 8% increased N-content in the rice plants and a strong plant growth promoting effect. Expression of *nif* genes is sensitive to high NH_4+ concentrations and so a genetically modified strain has also been generated with *nifA* or *nifA/ntrC* located on the bacterial transposon, Tn*5* (Smalla *et al.*, 2002). This has increased inoculant efficiency by reducing NH_4+ sensitivity.

It was not possible to obtain any data from the assays designed to test efficacy of the potential BCAs to suppress tomato late blight because no disease incidence

was established in the controls. In experiment **5.2.4.1** it is possible that the germinating seeds were exuding some type of antimicrobial substance which was inhibiting the pathogen. Exudates from germinating tomato seeds added to solarised soil increased populations of fluorescent pseudomonads but decreased fungal populations (Gamliel and Katan, 1992). It was still not possible, however, to establish disease two weeks after germination. Lack of disease incidence observed in plants maintained in the greenhouse may have been because it was not possible to maintain the moisture levels at the high levels vital for disease development (Ribeiro, 1983).

CHAPTER VI

Overall discussion / Future work

6.1 Variability of biocontrol

Owing to the huge environmental benefits and lack of success with alternative methods of disease prevention, biocontrol has been the subject of extensive research over the last three decades. However, this has not been reflected in the number of commercial successes (Paulitz, 2000, Handelsman and Stabb, 1996). This has been attributed to the failure of BCAs either to survive or to express the antagonistic properties revealed by *in vitro* screens. These characteristics are influenced by abiotic factors, including soil aeration, temperature, pH and texture, and biotic factors such as competition from and predation by other soil microbiota, pathogen strain and the crop cultivar (Shah-Smith and Burns, 1996 and Conn *et al.,* 1997). For example, the antibiotic 2,4-diacetylphloroglucinol, produced by a number of pseudomonads, is considerably more active *in vitro* against *Pythium* species at acidic than at neutral to basic pH levels (Raaijmakers *et al.*, 2002). Further laboratory, glasshouse and field trials would be required to assess the effect of these variables.

For biocontrol to provide greater success in plant disease protection, both in terms of the isolates selected in this investigation and in the wider context, it is necessary to develop a greater understanding of the environmental influences. Pierson and Weller (1994) examined the effectiveness of mixtures of fluorescent pseudomonads on the suppression of take-all of wheat. While strain combinations were found to be effective, they were unable to demonstrate consistency between sites of testing. This was probably attributable to large variations in soil and climate leading the authors to postulate that biocontrol of take-all may be achieved through the use of 10-15 core strains with several of the strains mixed depending on the soil type, the use of winter or spring wheat

cultivars, the levels of tillage and the inoculum potential of target and nontarget pathogens.

Ideally all 15 isolates originally selected in this investigation would be tested under a range of field conditions. This would increase the likelihood of selecting optimum BCAs as it is quite possible that laboratory results will not be accurately reflected in the field. For example, the length of time over which a BCA is effective at controlling disease is very important and cannot be predicted in the laboratory. Field trials, particularly in the context of the potato plant, are necessary (Sadfi *et al.,* 2002). However, it would cost £1000 to patent each bacterial isolate for the first year only (personnel communication D. Reay, 2003) and substantially more for subsequent years, rendering this option unfeasible.

The cultivar of host plant often affects the efficacy of the BCA (Handelsman and Stabb, 1996). Smith *et al.,* (1997) used population models and attempted to separate the effect of the host plant, in terms of its inherent disease resistance and its ability to support the BCA. Six different inbred lines of tomato were inoculated with one species of *Pythium* over a range of inoculum densities and different doses of the BCA, *Bacillus cereus* UW85. The responses of the cultivars to the pathogens were different from their responses to the BCA. There appears to be an inherent difference in the ability of a plant to support biological control, a genetic trait that may be bred and selected for (Paulitz, 2000). This is referred to as breeding for 'hospitality' in the host plant. Such plants may produce root exudates that support growth or induce expression of genes in the antagonist, attract the BCA to the infection site or respond to the BCA by ISR. The preliminaries of hospitality breeding have been achieved for tomato and *Bacillus cereus* UW85 by demonstrating heritability of the trait and mapping genes associated with hospitality. Inbred lines derived from a wide cross were assessed for their ability to support biocontrol by *B. cereus.* Substantial variation for the trait was observed among these lines, providing the basis for mapping the genes (Handelsman and Stabb, 1996).

6.2 Determining host root colonisation

Root colonisation is a characteristic frequently cited as being vital to the success of a BCA (Persello-Cartieaux *et al.,* 2003, Walsh *et al.,* 2001). This is can be difficult to assess, with many studies using the cultivation dependent method of

antibiotic resistant biocontrol isolates (de Weger *et al.,* 1987). Rifampicin resistant mutants (mutation of the ribosomal binding site) are most commonly used because of the low background level of indigenous soil bacteria resistant to this antibiotic (unlike kanamycin, streptomycin or gentamycin; Smalla *et al.*, 2002). The production of pseudomonads with antibiotic resistance is relatively easy and rifampicin resistance as a marker of *P. putida* in the field has been shown to be stable (Glandorf *et al.*, 1992). It is necessary to ensure that by selecting for antibiotic resistance no other trait is affected. This method has been used to demonstrate that colonisation abilities are not always vital to disease suppressing abilities. Studies with rifampicin-resistant strains of putative BCAs including a *Pseudomonas putida* strain revealed that long-term colonisation of the tuber surface in at least some circumstances may not be necessary to reduce potato silver scurf symptoms (Elson *et al.,* 1997).

Immunoassays have also been employed. For example, colonisation of *Botrytis cinerea* infected strawberry leaves by three strains of *Ulocladium atrum* was successfully measured using specific monoclonal anti-*U. atrum* antibodies (Berto *et al.,* 2001).

Studies of microbial communities and biocontrol associated characteristics such as root colonisation has been greatly facilitated by the use of combinations of the green fluorescent protein and its colour variants, cyan fluorescent protein, yellow fluorescent protein and DsRed as markers. Broad-host-range plasmids expressing these proteins can be used to study up to three, differently marked, bacterial populations on the root. Visualisation of gene expression in the rhizosphere will provide detailed information on the functioning of bacterial cells in a specific environment (Bloemberg and Lugtenberg, 2001). These techniques may also be used to study the effect of the BCA on the indigenous microbial community and vice versa which may contribute to an understanding of the interactions in the rhizosphere and therefore help reduce variable biocontrol. These types of investigations do, however, have associated time and cost limitations.

6.3 Dose-response relationships

The dilution series results (chapter V) represent preliminary information on the dose-response relationships of these BCAs. This relationship is important for

fully understanding the dynamics of the interactions between the antagonists and *P. infestans* and is crucial for predicting the success of a biocontrol strategy. Based on papers presented at the International Congress of Plant Pathology, Edinburgh, 1998, Paulitz (2000) summarised some key questions concerning population dynamics of soil-borne pathogens and BCAs, these included; how does the pathogen respond to different levels of the BCA? What effect does the pathogen population have on the BCA? What is the efficiency of the BCA in controlling the pathogen? What are the maximum levels of pathogen pressure under which the BCA can give adequate control? Further work is required to establish precisely the specific limits of control in relation to both *P. infestans* and the potential BCAs from this investigation.

Substantial progress has been made towards answering these questions in a number of biocontrol systems through the application of genetic and mathematical approaches that accommodate the complexity (Handelsman and Stabb, 1996). Johnson (1994) proposed a dose-response relationship model for biocontrol based on the same principles applied to the interactions between pathogens and host plants. The model predicts that the relationship between BCA and pathogen can become asymptotic, such that increased antagonist does not compensate for deficiencies in disease suppression. This has been demonstrated to be true for the biocontrol of crown gall of tomato and cherry by *Agrobacterium radiobacter*. This BCA antagonises, in part, owing to its ability to produce the antibiotic agrocin-84. Dose-response relationships were also investigated for pathogen populations resistant to this antibiotic and for a BCA deficient in its production. This resulted in relationships that were apparently asymptotic at levels of control significantly less than those achieved when the pathogen population was susceptible to and the BCA produced the antibiotic. This illustrates the potential for the incorporation of mechanism of inhibition into models of pathogen-BCA interaction (Johnson and DiLeone, 1999).

Raaijmakers *et al.,* (1995) studied the interactions of *P. putida* strain WCS358 (antagonises by siderophore production) and *P. fluorescens* strain WCS374 (antagonises by ISR) with Fusarium wilt of radish and found similar non-linear, asymptotic relationships. Absolute disease reduction reached a maximum of approximately 30% at an average level of disease incidence of 50% for both mechanisms. At moderate to relatively high levels of disease incidence for both mechanisms, a threshold population density of the bacterial strain of

approximately 10^5 cfu per gram of root is required for significant suppression of Fusarium wilt of radish. When rhizosphere population densities of both strains dropped below this threshold level, a relatively small decline in the population density had a major effect on their efficacy to control disease.

6.4 Determining dose-response relationships

For reliable and economical disease control it is important to determine these epidemiological relationships between pathogen population and inoculated antagonists. This is can be difficult with factors such as pathogen strain, environmental conditions, indigenous soil microbiota and host cultivar all likely to affect the dose-response relationships in a largely unpredictable manner. However, advances in molecular and biochemical techniques are facilitating these areas. The use of monoclonal antibodies in commercial ELISA detection kits, bioassays and baiting can be used to detect *P. infestans.* A variety of PCR based approaches have been applied to detection of *Phytophthora* species and recently ligase chain reaction (LCR) coupled with PCR has been developed for enhanced detection of *P. infestans* (Tooley *et al.*, 2002). Sequences of the internal transcribed spacer 2 (ITS2) of ribosomal DNA from 10 different species of *Phytophthora* were aligned, and regions of dissimilarity were used to construct ligase chain reaction primers for detecting the late blight pathogen. Of the 15 *Phytophthora* species tested differentiation of species down to a single base pair difference was possible. The LCR primers could also be labelled with biotin and dioxigenin and therefore combined with an ELISA-based detection system also providing increased sensitivity (Tooley *et al.*, 2002).

Bacteria can be studied *in vivo* using some of the techniques discussed above. Antibiotic resistant mutants, marker or reporter genes, including *luc, lux* (firefly and *Vibrio fischeri* luciferase), or *gfp* (green fluorescence protein of *Aequorea victoria*), can be used to tag BCAs allowing their rapid and unambiguous detection (Smalla *et al.*, 2002). Monitoring of genetically modified rhizobacteria is fairly straight forward using strain-specific PCR systems, however these are often not available for non-modified microbial inoculants. In these cases cultivation dependent (selective plating on antibiotic containing medium) combined with a cultivation independent approach (16S based DGGE fingerprints) has provided satisfactory results (Smalla *et al.*, 2002). Lin *et al.*, (2000) generated a strain specific probe from the hypervariable region of rice PGPR diazotroph *Alcaligenes*

faecalis which successfully monitored the population density of this BCA, although this approach was not sensitive enough to detect the BCA at low cell densities.

Antibiotic resistance and denaturing gradient gel electrophoresis (DGGE) were used to demonstrate that the BCA, *Pseudomonas chlororaphis* 24_4, which antagonises the potato pathogen *Ralstonia solanacearum,* has excellent rhizosphere competence, maintaining a presence of 10^7 cfu/g root material at three week post-inoculation. In addition a proportion of samples was incubated with bromo deoxyuridine after which DNA was isolated and analysed by DGGE. This provided evidence that the BCA remained metabolically active over the three weeks of the experiment. Similar techniques were also applied to the pathogen and this demonstrated that the relative abundance of the pathogen and its metabolic activity was severely reduced in the rhizosphere of tomato plants inoculated with *P. chlororaphis* 24_4 (Smalla *et al.*, 2002). These approaches are useful for monitoring introduced biocontrol populations, although it does appear that techniques with increased sensitivity are required.

In order to study in greater detail the interactions between pathogen, BCA and crop, it may be possible to exploit a system using potato microtubers. These can be grown in aseptic conditions on defined nutrient medium in a controlled environment, which may be more representative of the *in planta* situation than the use of tissue cultured explants (Coleman *et al.*, 2001).

6.5 Application of the biocontrol agents

Application of the BCA(s) may represent several problems. The BCA must remain viable in the application formulation or substrate and above a predetermined critical threshold prior to use. For example, *Pseudomonas aureofaciens* 63-28 is an effective BCA against *Pythium* and *Rhizoctonia.* An ideal product would be the bacterium formulated in a growing mix. However population studies showed that *P. aureofaciens* did not survive at a critical threshold of 10^6 cells/g mix for more than 2 to 3 months in peat at 45% moisture, the moisture level at which it is sold. Freeze drying or formulations in vermiculite gave survival rates of six months to one year, if stored dry. However, if added to the peat growing mix at 45% moisture, the populations declined in 2 to 4 months (Paulitz, 2000).

BCA of potato diseases operating at the tuber level are commonly applied by seed tuber bacterisation. Tanii *et al.,* (1990) undertook field trials of seed tuber bacterisation with BCAs of scab, black scurf and soft rot of potatoes. They concluded that this method of application has potential as a useful and promising method for controlling soil-borne, tuber-borne and storage diseases of potato. Treatment of potato seed pieces with fungicides effective against *P. infestans* contributed to an increase in plant emergence and stand uniformity and a decrease in the spread of the pathogen from seed piece to foliage. For maximum benefit the seed pieces had to be treated before coming into contact with the pathogen (Powelson and Inglis, 1999). This approach may be effective for the application of the BCAs selected in this study.

Some research has been undertaken into the application of *Pseudomonas putida* and *Pantoea agglomerans,* two of the three species selected in this investigation to patent and research further. Continuous application technology using a self contained field fermentor has been applied to *P. putida* with encouraging results. High populations of the BCA were maintained over three years and the target pathogen, *Phytophthora parasitica* declined in the third year in a well established orchard and after the second year in a newly planted orchard (Steddom *et al.,* 2002). As mentioned in chapter III Costa *et al.,* (2000, 2002) have been researching various methods of preserving and packaging *P. agglomerans.* The use of 10^{10} cfu/ml as an initial concentration, sucrose as a protectant and non-fat skim milk as a rehydration medium enabled 100% viability to be conserved after freeze-drying. Work has also been on going to establish methods and materials for producing *P. agglomerans* using commercial products and by-products with minimal cost (Costa *et al.,* 2001). Little research has been done on application or formulation of *Trichosporon* spp (isolate 7). Other yeasts have been examined further, for example *Candida oleophila,* marketed commercially as Aspire and used to control postharvest decay of citrus fruit. Commercial testing of preparations of this yeast has begun but stand-alone treatments did not reduce the decay to commercially acceptable levels. It is also possible that the principles of preparation used for this yeast may not be suitable for *Trichosporon* spp.

6.6 The potential for improved biocontrol by genetic modification

Genetic modification by introducing genes which confer or improve biocontrol ability holds substantial potential. For example, the introduction of the *sss* colonisation gene from *Pseudomonas fluorescens* WCS365 into other *Pseudomonas* biocontrol strains improved competitive colonisation ability of tomato root tips (Dekkers *et al.,* 2000). Albicidin phytotoxins are produced by the devastating disease of sugarcane known as leaf scald, caused by *Xanthomonas albilineans.* Zhang *et al.,* (1999) have successfully generated transgenic sugarcane plants which express an albicidin detoxifying gene (*albD*) originally found in *Pantoea dispersa.* Transgenic lines with high albicidin detoxifying enzyme activity in young stems were also protected against systemic multiplication of the pathogen. Similarly, if the gene encoding for the production of the compound responsible for the morphogenetic effect on *P. infestans* hyphae (Chapter IV), could be identified then potato cultivars could be engineered to express that gene. This may, however, place a selection pressure on the pathogen population resulting in the emergence of resistant genotypes. The application of a cultivar engineered to express this property, in conjunction with another effective BCA may avoid this problem and prove successful.

6.7 Marketing biocontrol

Marketing biocontrol products requires specialised knowledge of the target plant disease, the biological control agent, integrated disease control practices, production and storage systems and microbial ecosystems. Distributing both the product and the knowledge necessary for its successful use will be the only effective way to ensure long-term market acceptance (Janisiewicz and Korsten, 2002).

6.8 Next steps

If this investigation were to continue the immediate next steps would be 1) to isolate and identify the compound, produced by isolate 7, which is causing the hyphae of *P. infestans* to distort, 2) to establish dose-response relationships, as it is necessary to understand more precisely the relationship between numbers of propagules of each biocontrol isolate and the numbers of *P. infestans* propagules and the level of control obtained, 3) field trials would commence as soon as possible as it is vital to determine the sensitivity of the BCAs to environmental

fluctuations and the length of time and concentration at which the BCAs will persist and remain effective in the rhizosphere and 4) the isolates would also be tested against a variety of *P. infestans* strains.

Appendix I

All recipes are per one litre of deionised water unless otherwise stated.
All agar is bacteriological agar (Oxoid) and chemicals were obtained from Sigma, unless otherwise stated.
All media were autoclaved at 121°C for 15 min.

(5%) V8 Agar (Skidmore *et al.*, 1984)
300 ml V8 juice (Campbell Grocery Products, King's Lynn, Norfolk, UK)
20 g agar (Difco Labs, Detroit, Michigan, USA)
4g $CaCO_3$ (BDH, Poole, Dorset, UK)

V8 + β-sitosterol (V8S, Medina and Platt, 1999)
V8 agar as above
After autoclaving:
25mg β-sitosterol dissolved in 3ml 95% ethanol with two drops of Tween 80, diluted with 50ml sterile deionised water, and mixed with 940 ml cooled (45°C) V8 basal medium.

Rye A agar
60 g whole organic rye grains (Infinity Foods, Brighton, E Sussex, UK)
Grains washed briefly in tap water and then soaked for approximately 36 h at room temperature.
Supernatant poured off and retained.
Swollen rye grains macerated with hand-held food processor and then heated at 50°C for 3 h in enough deionised water to cover.
Grain mixture filtered through four layers of muslin and steel strainer and sediment discarded.
Original supernatant and grain extract combined with 15 g agar (Difco) and 20 g sucrose (BDH).

Antibiotics
20ml DMSO
0.5g rifamycin
0.5g ampicillin
2ml then required per litre of medium.
(antibiotics stored in freezer)

Potato Periderm

Potato peelings were dried in an oven.

Thoroughly dried periderm was then either powdered in a food processor or ground with a pestle and mortar.

Autoclaved at 121°C for 15 min

Appendix II

All recipes are per one litre of deionised water unless otherwise stated.
All agar is bacteriological agar (Oxoid) and chemicals were obtained from Sigma, unless otherwise stated.
All media were autoclaved at 121°C for 15 min.

Non-selective media

Nutrient agar (NA)
28g nutrient agar

1/10 Nutrient agar
2.8g nutrient agar
13.5g agar

Tryptic soy agar (TSA)
40g tryptic soy agar

1/10 Tryptic soy agar
4g tryptic soy agar
15g agar

Growth medium mimicking rhizosphere (Buyer *et al.*, 1989)
0.75g $Ca(NO_3)_2 \cdot 4H_2O$
0.246g $MgSO_4 \cdot 7H_2O$
18.22g ACES (2-[(2-Amino-2-oxoethyl)-amino]ethanesulfonic acid)
2g NaOH
were dissolved in 853 ml deionised H_2O
For the plates 15g agar was added.
After autoclaving, the following sterile stock solutions were added
1 ml 1 *M* KH_2PO_4 pH 7
1 ml 7 x 10^{-4} *M* $ZnSO_4 \cdot 7H_2O$
1 ml 9 x 10^{-4}*M* Mn $SO_4 \cdot 4H_2O$
1 ml 20 mg/l thiamine HCl
1 mg/l biotin
100ml 10% casamino acids

33.3ml 30% sucrose

The phosphate, casamino acids and sucrose were autoclaved separately while the metal mixture and vitamins were filter-sterilised.

Selective Media

King's B medium basal formulation, with selective media formulation of novobiocin-penicillin-cycloheximide (KB, King *et al.,* 1954)

Full strength

20g peptone

1.5g K_2HPO_4

1.5g $MgSO_4 \cdot 7H_2O$

10g Na succinate

15g agar

pH 7.2

KB/250

Same as above with 250-fold dilution of glycerol and peptone and the mineral salts as 10-fold dilutions.

Selective formulations (Sugimoto *et al.,* 1990)

45 mg novobiocin

75 000 units penicillin

75mg cycloheximide per litre

These agents were dissolved in 3 ml 95% ethanol, diluted with 50 ml sterile distilled water, and mixed with 940 ml autoclaved and cooled (45°C) KB basal medium, diluted 250-fold for that strength.

1/10 Tryptic soy agar with crystal violet

as above with 0.005 g/l crystal violet

Potato dextrose agar (PDA) containing rifamycin and ampicillin antibiotics

39g PDA

Antibiotics as for *P. infestans* isolation

***Trichoderma* selective medium** (T.S., adapted by Etebarian *et al.,* 2000)

3g glucose

0.15g KCl

0.2g $MgSO_4.7H_2O$

1g NH_4NO_3

0.9g K_2HPO_4

15g agar

After autoclaving add

250mg chloramphenicol

10mg metalaxyl

200mg pentachloronitrobenzene (Terraclor, Olin chemicals, USA)

150mg rose bengal

***Bacillus subtilis* selective medium** (B.S.S., Turner and Backman, 1991)

400ml V8 juice

40g NaCl

1g dextrose

20g agar

600ml deionised water

pH adjusted to approximately 5.2 before autoclaving

Selective medium for *Pseudomonas cepacia* (P.C.S., Hagedorn *et al.,* 1987)

20g agar

2g glucose

1g L-asparagine

1g $NaHCO_3$

500mg KH_2PO_4

100mg $MgSO_4.7H_2O$

50mg trypan blue

20mg tetracycline

The pH was adjusted to 5.5 with 10% phosphoric acid (4ml/l) and the filter-sterilised tetracycline was added to the autoclaved medium.

For lower soil dilutions in which the fungi were numerous, crystal violet (5mg/l) and filter-sterilised nystatin were added.

Water-yeast extract-agar (WYE, Crawford *et al.*, 1993)

0.25g yeast extract

18g agar

0.5g K_2HPO_4

Adjusted to pH 7.1 – 7.2 with 0.1 *N* NaOH prior to autoclaving

Casamino acids-yeast extract-glucose-agar (YCED, Crawford *et al.*, 1993)

0.3g yeast extract

0.3g casamino acids

0.3g D-glucose

18g agar

0.2g K_2HPO_4

Adjusted to pH 7.1 – 7.2 with 0.1 *N* NaOH prior to autoclaving

Peptone-rosebengal (P-R)

5g peptone

10g dextrose

20g agar

1g KH_2PO_4

0.5g $MgSO_4.7H_2O$

0.03g Rose Bengal

After autoclaving 0.03g streptomycin added to media held at 50°C in water bath.

Yeast malt extract agar (YME)

20g agar

10g glucose

5g peptone

3g malt extract

3g yeast extract

***Bacillus brevis* selective medium** (B.B.S., Edwards and Seddon, 2000)

6.5g nutrient broth

5g tyrosine

15.0g agar

Enrichment Solutions

***Pseudomonas fluorescens* enrichment medium**

5g NaCl

0.2g $MgSO_4$

1g $NH_4H_2PO_4$

1g K_2HPO_4

2g Na Benzoate

pH 6.8

Ringers Solution

1 Ringers solution tablet (BDH) per 500ml deionised water

Appendix III

All recipes are per one litre of deionised water unless otherwise stated.
All agar is bacteriological agar (Oxoid) and chemicals were obtained from Sigma, unless otherwise stated.
All media were autoclaved at 121°C for 15 min.

Phosphatase activity

To test for both acidic and alkaline phosphatases ;

Filter sterilised 1% solution of sodium salt phenolphthalein diphosphate was added to autoclaved nutrient agar to give a final concentration of 0.01%. Streaked plates were incubated for 2 to 5 days. 1 drop of ammonia solution (28 – 30%) was placed in the lid of an inverted plate and the culture replaced over it to allow the ammonia fumes to reach the colonies. A positive result was indicated by red colonies, as a result of the presence of free phenolphthalein.

Gelatine hydrolysis (Kloepper *et al.,* 1991).

Gelatine plates were inoculated and incubated for 10 days at 28°C, then placed at 4°C for 1hr. Liquefaction of the semi-solid gelatine medium indicated a positive reaction

Gelatine medium (U.S. FDA, 2001)
4g peptone
1g yeast extract
15g gelatine
15g agar

Catalase Test

10% hydrogen peroxide was added to cells on a slide. Effervescence was considered catalase positive.

Oxidase Test

Filter paper was moistened with 1% tetramethyl phenylene diamine in 0.1% ascorbic acid and used to rub a bacterial colony. A purple colour in 10-15 seconds was considered oxidase positive.

Production of HCN

TSA (see appendix 2) supplemented with 4.4g L^{-1} glycine

Inoculate medium with test colonies and place filter paper strips soaked in picric acid solution (2.5g picric acid, 12.5g Na_2CO_3) in the lid of the petri dish. The dishes were sealed with parafilm and incubated for 2-4 days. HCN production was indicated by a change in the colour of the filter paper from yellow to reddish brown.

Lecithinase activity

A plate of egg yolk agar was streaked to obtain well isolated colonies. A positive test is indicated by a cloudy (opaque) zone within the medium around the colony

Egg yolk agar;

Per 500ml deionised water

20g peptone

2.5g Na_2HPO_4

1.0g NaCl

0.1ml $MgSO_4$, 0.5% (wt/vol) solution

1.0g glucose

12.5g agar

Combine and adjust pH to 7.3-7.4. Autoclave. Disinfect surface of egg, add egg yolk aseptically to 60°C agar and mix.

Cellulolytic activity (Alstrom, 2001).

Bacteria were grown for 2-5 days on cellulose containing nutrient medium;

20g tryptic soy broth

15g agar

5g Na-carboxymethylcellulose

The plate was then flooded with 0.1% congo red stain prepared in distilled water for 15min, following destaining with 1M NaCl for 15min. Appearance of a yellow halo indicated a positive response.

Proteolytic activity (Alstrom, 2001)

Measured as formation of clear zones in agar plates containing;

15g skimmed milk powder

15g agar

Starch hydrolysis (Kloepper *et al.*, 1991)

Inoculated starch agar plates were flooded with gram stain iodine after 48hr incubation at 28°C, starch hydrolysis was indicated by clear zones surrounding the bacteria.

Starch agar (U.S. FDA, 2001)

23g nutrient agar

10g potato starch

Oxidation of phenol (Kloepper *et al.*, 1991)

Bacteria were grown on gallic acid agar;

15g agar

2g $NaNO_3$

0.5g $MgSO_4.7H_2O$

1g KH_2PO_4

2mg thiamine HCl

425mg gallic acid

Presence of growth was indicated by a visible lawn within 7 days of inoculation.

Lipid Hydrolysis

Bacteria were grown on nutrient agar supplemented with 0.01% $CaCl_2.H_2O$ and sterilised Tween 80 (oleic acid ester). The tween were added to autoclaved molten agar to give a final concentration of 1% (vol/vol). The medium was shaken until the tween was completely dissolved.

An opaque halo around the colony was a positive result.

Tryptophan hydrolysis (Indole test)

1% tryptone broth;

10g tryptone

5g NaCl

was inoculated and shaken (100 rpm). Cultures of different ages were tested.

5ml of bacterial culture was extracted into a tube and 1ml of water-saturated toluene was added, then the culture agitated vigorously. The toluene was then allowed to form a layer at the top of the broth. Ehrlich's reagent (0.5ml) was then trickled down the wall of the tube to form a layer between the toluene and the

broth. The tube was allowed to stand for 5 minutes. A red ring just below the toluene layer was a positive response. The control was a tube of uninoculated medium.

Ehrlich's reagent;

1g *p*-Dimethylaminobenzaldehyde

95ml ethanol

20ml conc. HCl

Dissolve the aldehyde in the ethanol, add the acid and store in the fridge, protected from light.

Appendix IV

All recipes are per one litre of deionised water unless otherwise stated.
All agar is bacteriological agar (Oxoid) and chemicals were obtained from Sigma, unless otherwise stated.
All media were autoclaved at 121°C for 15 min.

CD-V8 Liquid Medium
45.5g Czapek-Dox (Oxoid, Unipath Ltd., UK)
1g mycological peptone
1g yeast extract
1g casein hydrolysate
supplemented with 200ml of clarified V8 juice (Campbell Grocery Products Ltd., UK) prepared by boiling the juice, filtering through 4 thicknesses of muslin and centrifuging at 10 000*g* for 5 minutes. The medium was then distributed into 250ml Erlenmeyer flasks (100ml per flask) and autoclaved.

Minimal Liquid Medium (MLM, Eckwall and Schottel, 1997)
15mM $(NH_4)_2SO_4$
2mM $MgSO_4 \cdot 7H_2O$
15mM NaH_2PO_4/K_2HPO_4 pH 6.8
3.5μM $ZnSO_4 \cdot 7H_2O$
3.6μM $FeSO_4 \cdot 7H_2O$
5.1μM $MnCl_2 \cdot 4H_2O$
9μM $CaCl_2$
0.5% casamino acids
1% (w/v) glucose

Chrome Azurol S agar (CAS, Schwyn and Neilands, 1987)
10 x MM9
3g KH_2PO_4
5g NaCl
10g NH_4Cl

CAS-HDTMA (500ml water)
650mg Chrome azurol S

Dissolve. Add 100ml 1mM $FeCl_3 \cdot 6H_2O$ in 10mM HCl. Add slowly to hexadecyltrimethylammonium bromide (HDTMA) solution (729 mg in 400ml water)
Autoclave.

Deferrated casamino acids
Dissolve 10g casamino acids in 100ml water. Extract the casamino acids solution with an equal volume of 3% 8-hydroxyquinoline in chloroform to remove contaminating iron. Extract with an equal volume chloroform to remove traces of 8-hydroxyquinoline.

CAS agar (750ml water)
6g NaOH
30.24g PIPES
100ml 10 x MM9
15g agar
Autoclave. Cool to 50°C and add
30ml deferrated casamino acids
10ml 20% glucose
1ml 1 M $MgCl_2$
1ml 100 mM $CaCl_2$
Mix. Add 100ml CAS-HDTMA solution, mix gently to avoid foaming. Pour plates.

Modified CAS agar (Alexander and Zuberer, 1991)
Solutions sterilised separately before mixing

Fe-CAS indicator solution
10ml 1mM $FeCl_3 \cdot 6H_2O$ in 10mM HCl
50ml CAS aqueous solution (1.21 mg ml^{-1})
mixed and added slowly, with constant mixing to
40ml HDTMA aqueous solution (1.82 mg ml^{-1})
Autoclave.
All the reagents in the indicator solution were freshly prepared for each batch of CAS agar

Buffer solution

Dissolve 30.24g PIPES in 750ml of a salt solution;

0.3g KH_2PO_4

0.5g NaCl

1g NH_4Cl

pH was adjusted to 6.8 with 50% KOH and water was added to bring the volume to 800ml

15g agar

autoclave and cool to 50°C

Minerals and carbon

2g glucose

2g mannitol

493mg $MgSO_4 \cdot 7H_2O$

11mg $CaCl_2$

1.17mg $MnSO_4 \cdot H_2O$

1.4mg H_3BO_3

0.04mg $CuSO_4 \cdot 5H_2O$

1.2mg $ZnSO_4 \cdot 7H_2O$

1mg $Na_2MoO_4 \cdot 2H_2O$

Autoclave and cool to 50°C then add to buffer solution with

30ml filter-sterilised 10% (w/v) casamino acids

The indicator solution was added last with gentle stirring.

Modified M9 liquid medium

The same as modified CAS agar except the agar and indicator solution were omitted.

Modified CAS assay solution

21mg HDTMA was dissolved in 25ml of water over a low heat

Separately 1.5ml 1mM $FeCl_3 \cdot 6H_2O$ (in 10mM HCL) was mixed with 7.5ml of 2mM CAS.

This solution was slowly added to the HDTMA solution while stirring and the mixture was transferred to a 100ml flask.

The buffer was prepared by dissolving 9.76g MES in 50ml water. The pH was adjusted to 5.6 with 50% KOH, and the buffer solution was added to the indicator solution. Water was added to bring the volume up to 100ml.

Reagents for measuring ß-1,3-glucanase activity (Dygerts *et al.*, 1965)

Reagent A

dissolve 40g anhydrous Na_2CO_3 in 600ml dH_2O

16g glycine and dissolve

0.45g $CuSO_4.5H_2O$ and make up to 1 litre with water

Reagent B

dissolve 0.12g of neocuproine.HCl in 100ml dH_2O

Protease inducing medium (Elad and Kapat, 1999)

1.4g $(NH_4)_2SO_4$

2g KH_2PO_4

6.9g NaH_2PO_4

0.3g $MgSO_4 \cdot 7H_20$

1g dextrose

10g peptone

0.3g urea

The pH was adjusted to 5.0 using 1 N HCL

Holding buffer

10.5g citric acid monohydrate

0.247g $MgSO_4 \cdot 7H_20$

0.174g K_2HPO_4

0.735g $CaCl_2 \cdot 2H_20$

6.2g NaOH

100g glucose

acidified to pH 6.4 using 1 M HCL

Appendix V

Meetings at which PhD research presented:

- The Perry Foundation, Chelmsford, 02/2002.
- Conference on Global Food Security, Imperial University, London, 06/2002.
- London Area Molecular Plant Sciences, University College London, 09/2002.
- International Conference of Plant Pathology, Christchurch, New Zealand, 02/2003.
- PhD talk, University College London, 05/2003.

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